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Release Kinetic Profiles of 6-Mercaptopurine Loaded Covalently Functionalized Multiwalled Carbon Nanotubes

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ABSTRACT

Most of the existing anticancer drugs are very potent small molecules; their efficacy is constrained by their systemic toxicity, narrow therapeutic window, low drug loading, size control, scale up, cost of formulation but also as a result of drug resistance and limited cellular entry. MWCNTs (Multiwalled Carbon Nanotubes) offers great advantages over existing delivery vectors, because the high surface area provides multiple attachment sites for drugs. In the present study, 6MP (6-Mercaptopurine) loaded CNTs are developed by solvent method; as a carrier for drug targeting to cancer tissues for exhibiting antineoplastic activity. Non-covalent functionalization of Multiwalled Carbon Nanotubes (MWCNTs) was achieved using basic treatment followed by treatment with HCL. The loaded nanotubes were shown to release the drug for more than 10h, thus controlling the release and the amount of drug released from the best performing formulation was 59.2%. The release was found to follow the zero-order release pattern. Our work established a novel, easy-to-make formulation of MWCNTs with better drug loading efficiency increases dispersibility of CNTs in water and bioavailability at cancer site with reduction of systemic toxicity provides new directions for preparation of efficient drug carriers.

Keywords: Multiwalled Carbon Nanotubes, 6-Mercaptopurine, Anticancer, Solvent Method.

INTRODUCTION

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 and

peptides[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/dispersed 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) are reported[14-18]. In the present study, 6MP (6, 7-dihydro-3H-purine-6-thione) (Fig 1) loaded CNTs are developed by slight modification in solvent method[19]; exhibiting antineoplastic activity. Non-covalent functionalization of Multi walled Carbon Nanotubes (MWCNTs) was achieved using basic treatment followed by treatment with HCL. Functionalized MWCNT were attached with 6MP by sonication in a suitable media (pH 7.4). The formulation was characterized for drug entrapment, pH and *in vitro* drug release study.

EXPERIMENTAL

Materials

MWCNTs with 10-15 nm outer diameter, 2-6 nm inner diameters and 0.1-10 µm length were purchased from Redex Technologies, Pvt. Ltd., Noida, India.6-Mercaptopurine (6MP) was received as a gift sample from the Dabur Pharmaceuticals, Baddi, India. All other chemicals were of analytical reagent (AR) grade.

Methods

Functionalization of Carbon Nanotubes

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

- Treatment with concentrated hydrochloric acid

This method simply purifies the CNTs. In this method 500mg of MWNCT was placed in a 500mL round bottomed flask and 200mL of HCL was added. The mixture was stirred using magnetic stirrer for 2h, then diluted in water, filtered, washed with ultrapure water and dried in vaccum at 40^{o}C overnight.

- Acidic treatment followed with hydrochloric acid

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 12h at room temperature. The MCWNTs were thoroughly washed with ultrapure water and dispersed in HCL and refluxed for 24h, further it was collected by filtration, washed with ultrapure water and dried in vacuum at 40^{o}C overnight.

- Basic treatment followed with hydrochloric acid

500mg of MWCNT was dispersed in 25mL of the mixture of ammonium hydroxide (25%) and hydrogen peroxide (30%) (V: V=1:1) in a 100 mL round bottomed flask equipped with a condenser and the dispersion was heated to 80°C and kept for 5h. Resulting dispersion was diluted in water, filtered, further washed with ultra pure water and the sample was dried in vacuum at 40°C overnight.

Optimization of the best method for functionalization of MWCNTs

Selection was made on the basis of dispersion stability. For this 10mg of functionalized nanotubes were dispersed into 10mL of phosphate buffer solution (pH 7.4) by sonication (Ultrasonic Bath Sonicator; HICON, New Delhi) for 2 minutes and dispersions were kept in sealed vials. The dispersion stability was visually analyzed after a period of 15 days. The best method MWCNTs were used for drug-loading and characterized by the use of FTIR spectroscopy (Perkin Elmer, US, Model No Perkin Elmer Spectrum II).

Preparation of drug loaded carboxylated MWCNTs

A mixture of 6MP (100mg) and carboxylated-MWCNTs was prepared in accordance to the ratio in the formulation (Table 1) and added to 10 mL of two different solvents 01.N NaOH and ethanol: water (1:9). The solution obtained was agitated for 4 hrs using ultra sonicator, and dispersion was filtered using vacuum filtration assembly fitted with membrane filter (0.45µm; Sigma Aldrich, Germany) and the residue was washed with ultrapure water. The product was finally dried at 40°C for 24 h.

Evaluation of 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 for determination of drug entrapment. The entrapment was determined by dispersing accurately weighed quantity of formulation (containing amount of drug equivalent to 50 mg), into 100mL of phosphate buffer (pH 7.4) and heating up to 37°C, to ensure the release of the entrapped drug. Aliquot of 1mL was withdrawn and further diluted to 10 mL with buffer, 6MP concentration was determined at 320nm by using UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan, Model No UV-1700 Pharma Spec).

In vitro Release Studies

The *in vitro* release of 6MP from all the formulations was studied through a dialysis membrane (Sigma Aldrich, Germany) with molecular weight cut off 12000. 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 100mL of phosphate buffer placed in the shaking water bath (HICON, New Delhi) and maintained at 37°C with a frequency of 50 shakings per minute. Aliquots, each of 5mL volume were withdrawn at regular intervals and replaced by an equal volume of the dissolution medium. The aliquots were then suitably diluted 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.

To find out the mechanism of drug release, 60% drug of release data was first fitted in the Korsmeyer-Peppas model. 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, Fickian mechanism governs drug release; if between 0.45 to 0.89, 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

The CNTs were functionalized as per the three methods; treatment with concentrated hydrochloric acid, acidic treatment followed with hydrochloric acid, basic treatment followed with treatment with hydrochloric acid. This selection was made on the basis of dispersion stability. For this 10mg 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 basic treatment followed by treatment with hydrochloric acid, as shown in Fig 2 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 are shown in Fig 3b. The peaks for carboxy group shows at1661$cm^{-1}$and hydroxyl group at 3432cm^{-1} which are absent in pristine MWCNTs in Fig 3a, thus proving that the MWCNTs are now carboxy functionalized.

Incorporation of drug by solvent method

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

Evaluation

Entrapment

Fig 4 shows the percent entrapment of drug for the formulations. The entrapment was found to be good since in solvent method a physical mixture of the drug and CNTs is first prepared, prior to incorporation using solvent, which allows better interaction between the drug and CNTs thus showing higher entrapment. Entrapment for the formulations F1, F2, F3, F4, F5 and F6 was found to be 38.73%, 47.19%, 50.01%, 40.19%, 47.68% and 50.55% respectively. The entrapment values were found to reach a maximum at around 50% and further increase in ratio of drug: CNTs showed no increase in entrapment. The low entrapment values can be compensated by the fact that CNTs would provide tumor targeting and hence the dose required would be lowered further.

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 6MP were subjected to *in vitro* release studies 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, after 10h were 69.6%, 56.2%, 68%, 51.3%, 59.2% and 47.8% respectively.

The F3 and F1 showed quite similar pattern of release during the first 5h. In this duration F3 showed a slight burst release in the first hour followed by a linear pattern of release afterwards, while F1 showed a slow initial release in the first hour, followed by a linear pattern in the entire period of 5h. F1 showed significantly less drug release as compared to F3. F2 on the other hand showed a slow initial release in the first 90 minute, followed by burst release till the 4h and this was followed by a linear release pattern. The formulation F5 and F6 showed an almost linear pattern of release; however the rate of drug release from F6 was much slower than F5. Formulation F4 also showed an almost linear pattern of release except for the period between 3 to 5h in which it showed slight burst release. Overall these 6 formulations prepared by the solvent method released lesser drug content within 10h, thus were found to be more suitable for controlled release, especially formulation F2, F4 and F6.

The *in vitro* release data indicates that the formulations prepared by the solvent method give more prolonged release. This was expected and this occurs due to the premixing of the drug with CNTs, allows time for more interaction to occur and thus aids in providing higher drug entrapment and also aids in providing controlled release. 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. The prolonged release in the later stage can be attributed to the slow release of the drug form the CNTs. The *in vitro* 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 6MP in blood. However, the results clearly show that the formulations, particularly the ones prepared by solvent method have the ability to release the drug for prolonged period of time 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 for the formulations were plotted and the regression coefficient (r^2) values and the 'n' values for Korsmayer pappas are tabulated in Table 3.

The Table 3, shows that in case of formulations prepared by solvent method, the best fit model was Zero order, while 'n' exponent value, for Pappas model, for formulations F3 and F6 is greater than 0.45 indicating that formulation is released by Non-Fickian diffusion mechanism. While for formulations F1, F2, F4 and F5 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 CNTs is a new strategy with the potential to maximize the efficacy of drug and reduce systemic toxicity. In this study, we have demonstrated the effectiveness 6MP using CNTs, thus increasing bioavailability at cancer site and reduction of systemic toxicity. However some more further studies 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-make formulation of MWCNTs with better drug loading efficiency. Group and increase

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 solvent 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 (n=3)

Time (hrs)	Formulation					
	Mean % cumulative release					
	F1	F2	F3	F4	F5	F6
0.08	0%	0%	3.3%	0%	0.3%	1.1%
0.25	1.5%	0.2%	7.3%	0.9%	1.1%	3.5%
0.5	3%	1.6%	9.9%	3.5%	3.2%	5.1%
1	6.1%	3.9%	12.9%	7.2%	6.2%	6.9%
1.5	11.6%	9.7%	15.1%	8.5%	10.2%	8.8%
2	15.4%	12.9%	18.1%	12.2%	13.4%	11.9%
3	21.6%	18.7%	24.2%	16.1%	19.7%	15.8%
4	28.4%	21.5%	31.2%	22.9%	24.5%	19.6%
5	35.4%	27.2%	36.7%	25.2%	29.2%	25.8%
6	42.1%	32.9%	42.9%	30.1%	35.6%	28.8%
7	48.1%	39.1%	48.7%	35.2%	40.8%	34.7%
8	55.2%	45.2%	55.5%	42.2%	47.7%	38.4%
10	69.6%	56.2%	68%	51.3%	59.2%	47.8%

Table 3. Regression Co-efficient for various models for the prepared formulations

Formulation	r^2				Korsmayer		Best Fit model	Release mechanism
	Hixson crowell	Zero	Higuchi	First	r^2	n		
F1	0.989	0.998	0.958	0.974	0.994	0.281269	Zero	Fickian
F2	0.991	0.996	0.955	0.984	0.946	0.111014	Zero	Fickian
F3	0.989	0.997	0.957	0.976	0.970	0.540977	Zero	Non Fickian
F4	0.992	0.995	0.958	0.987	0.976	0.304906	Zero	Fickian
F5	0.993	0.998	0.959	0.984	0.992	0.19627	Zero	Fickian
F6	0.995	0.998	0.953	0.991	0.981	0.45134	Zero	Non Fickian

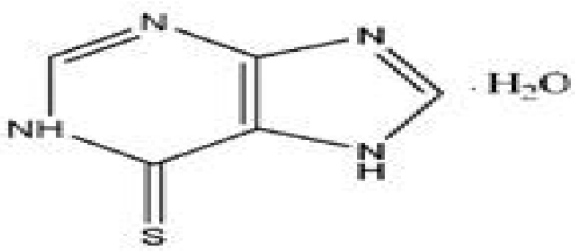

Figure 1. Molecular Structure of 6-Mercaptopurine

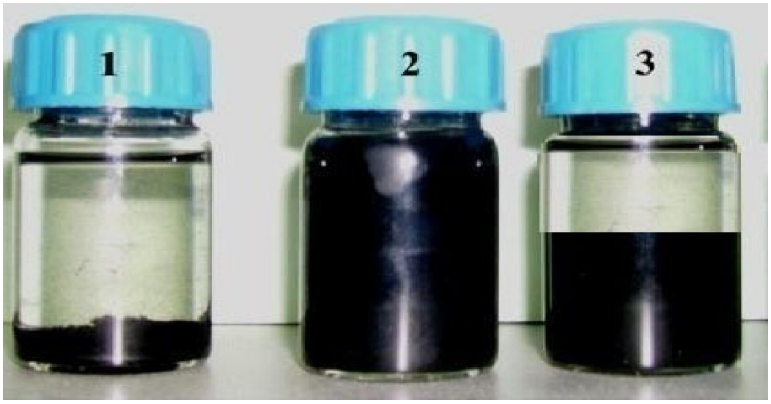

Figure 2. Dispersions of CNTs functionalized by different methods in phosphate buffer pH 7.4 (1=HCl treatment, 2=basic treatment and 3=acidic treatment) (picture taken after 15 days)

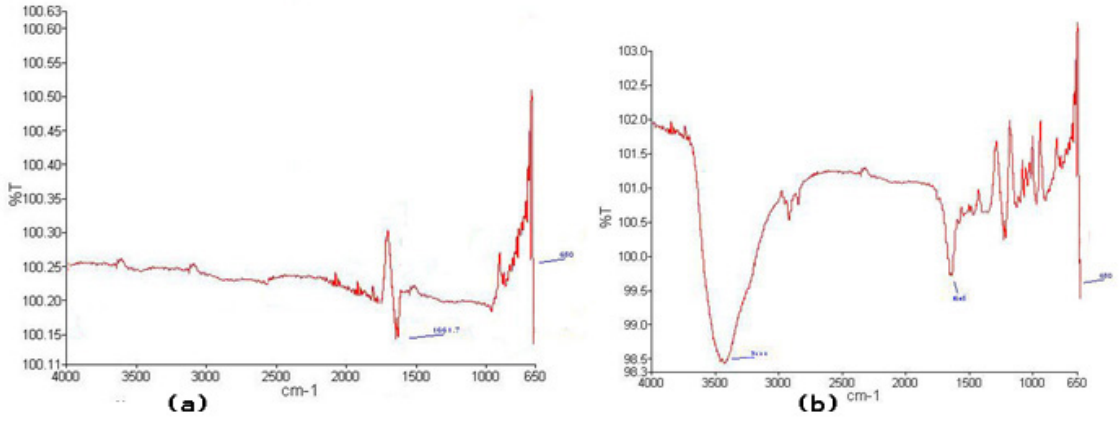

Figure 3. FTIR spectra of (a) pristine (untreated) MWCNT and (b) carboxylated MWCNT

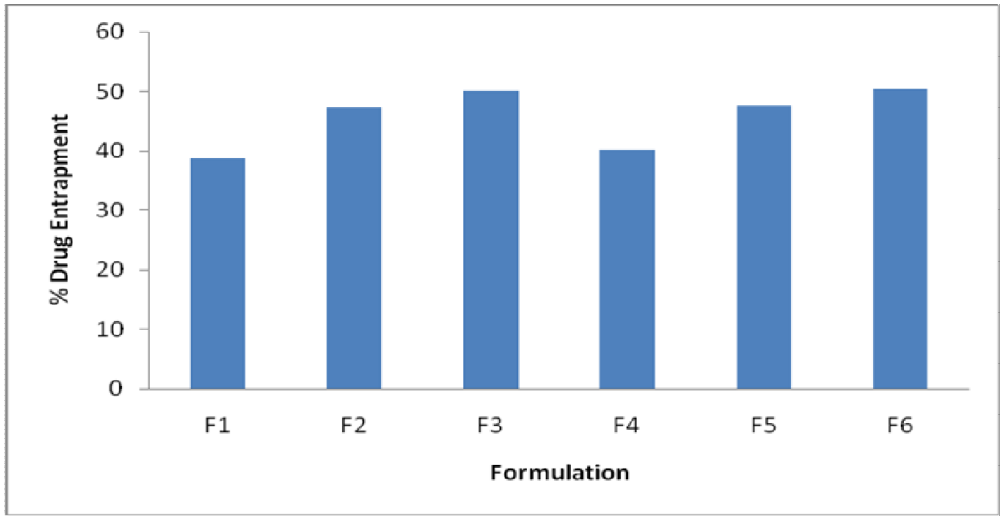

Figure 4. Percent Drug Entrapment of the prepared formulations

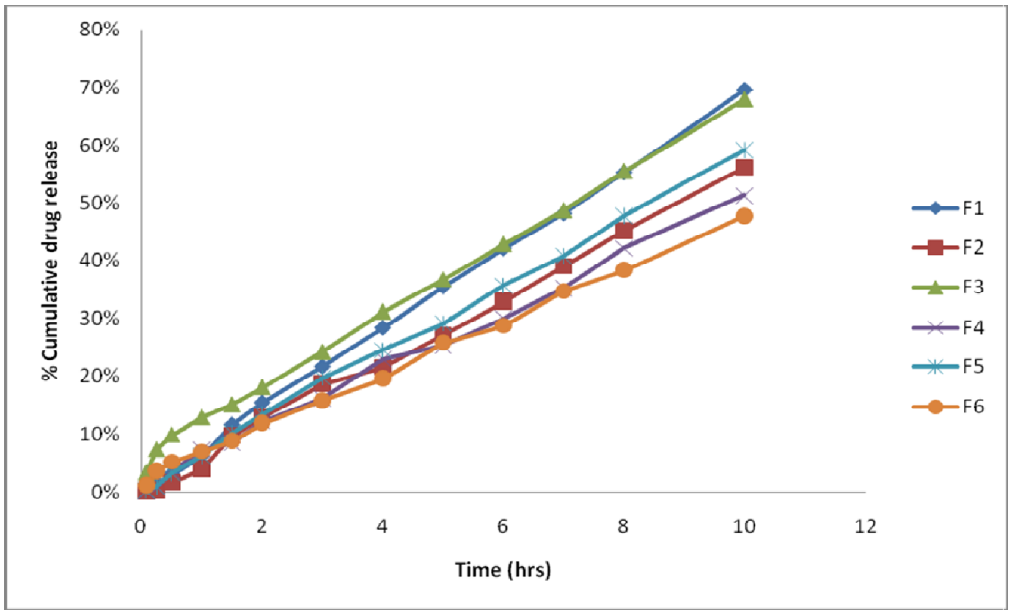

Figure 5. Release pattern of the prepared formulations