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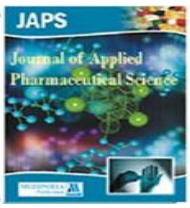

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Functionalization and evaluation of PEGylated Carbon Nanotubes as novel Drug delivery for methotrexate

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ABSTRACT

Several delivery systems are developed to target methotrexate to cancer tissues with limited success due to low drug loading, size control, toxicity, and scale up and also the cost of formulation. Off late, carbon nanotubes have been projected as a promising carrier for many drugs including anticancer agents. The present work is an attempt to investigate the potentialities of multi-walled carbon nanotubes (MWCNT) as a carrier for targeting methotrexate to cancer tissues. MWCNTs were functionalized using DSPE-mPEG 2000 and was then reacted with methotrexate (MTX) to produce MWCNT-mPEG-MTX conjugate. The conjugate was characterized for particle size, loading efficiency, morphology & rate of drug release. The result indicated that about 2.26 mg of Methotrexate per mg of MWCNTs were loaded with 56.5% entrapment efficiency. Particle size of the MWCNT conjugate was found to be less than 200nm with polydispersity index of 0.286 post lyophilization of the product. The MWCNT conjugate was found to release the drug faster in acidic medium than at neutral pH. However, in both neutral and acidic media, the release was continuous over the period of 48 hours.

Key words: Multiwalled Carbon Nanotubes, Methotrexate, DSPE-mPEG, Non-Covalent

INTRODUCTION

Treating cancer is a challenge because cancer chemotherapeutic agents are cytotoxic and could rarely differentiate cancer cells from normal cells. This leads to the destruction or impairment of vital organs in addition to killing cancer cells even at therapeutic dose levels, if their bio-distribution is not properly controlled and the therapeutic agents not targeted towards the cancer cells or tissues. Even though many targeted therapeutic systems are developed for anticancer drugs using vesicular and particulate carriers, their success is still limited due to several problems like low drug loading, size control, toxicity, scale up and also the cost of formulation (Ji et al., 2010). In attempts to investigate a suitable carrier system capable of taking reasonably high drug payload with targeting capability, carbon nanotubes are investigated by several workers (Bianco et al., 2005, Klumpp et al., 2006,Liu et al., 2008) for targeted delivery of many anticancer drugs (Srinivasan, 2008). In our present communication, methotrexate loaded carbon nanotubes are developed and were evaluated for drug loading & in-vitro diffusion study in acidic media.

Methotrexate is an anticancer drug belonging to the category of dihydrofolic acid reductase inhibitor used in the treatment of certain neoplastic diseases, severe psoriasis, and adult rheumatoid arthritis. It is used generally in higher doses for cancer treatment than required for other disorders and is often administered intravenously or intramuscularly. The drug's utility is limited due to its high systemic toxicity. Targeted anti cancer delivery systems containing

methotrexate are reported by several workers (Wanichsiriroj et al., 1985, Woo et al., 1983, Vodovozova et al., 2007, Kim et al., 1994) with drug loading capabilities varying from 12 % to 80 % based on the components of the delivery system and their methods of preparation.

The carbon nanotubes are allotropes of carbon with nanostructure having length to diameter ratio greater than 1 million. They have hexagonal network of carbon atoms woven into hollow cylinders having diameters as small as 0.7 nm and reach several millimeters in length (Annabelle, 2004). There are 6 types of carbon nanostructures 1) single walled, 2) multiwalled, 3) nanoflower, 4) nanobud, 5) fullerites and 6) torus. Multiwalled carbon nanotubes (MWCNT) have diameter up to 20 nm (I. Sumio, 1991) and are much studied for a variety of applications. Carbon nanotubes as such are highly hydrophobic and not dispersible in aqueous systems. Functionalization of carbon nanotubes 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 RES uptake (Prato et al., 2008, Jaffe, 2005). In addition, carbon nantoubes have other important properties like ultra high drug loading capacity, selective drug release in acidic environment etc.

The work communicated in this paper include the development of the Methotrexate loaded PEGylated carbon nanotubes. Non-covalent functionalization of Multi walled Carbon Nanotubes (MWCNTs) was achieved using DSPE-mPEG for PEGylation. Functionalized MWCNT were attached with methotrexate by sonication in a suitable media (pH 7.4). The formulation was characterized for particle size, drug entrapment and surface morphology. The samples were lyophilized and stability was assessed. In-vitro diffusion studies were conducted in acidic medium (pH 5.8) to evaluate its targeting potential.

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 Sigma-Aldrich, Germany, 1,2 - Distearoyl-phosphatidylethanolamine-methoxy-polyethylene glycol conjugate -2000 (DSPE-mPEG 2000) was received as gift sample from the Sun Pharma Advanced Research Centre, Baroda, India, Methotrexate was received as a gift sample from the Zydus Cadila, Ahmedabad, India. All other chemicals were of laboratory grade purchased from local suppliers.

METHODS

Preparation of drug loaded PEGylated MWCNTs

MWCNTs were functionalized by mixing it with DSPE-mPEG 2000 in water and sonicated in a bath sonicator. Unbound surfactant was thoroughly removed by repeated filtration through 100 kDa filters (Millipore). The functionalized MWCNTs were then resuspended in phosphate-buffered saline (PBS) by sonication in a bath sonicator and were mixed separately with the known concentration of methotrexate solution prepared in different buffer. The mixture was kept overnight at different pH conditions. Process parameters like sonication time and drug loading time were optimized on the basis of particle size and drug entrapment respectively. Ratio of MWCNTs: DSPE-mPEG: MTX was also optimized on the basis of particle size and drug entrapment efficiency (Chen et al., 2001, Britz et al., 2006).

Characterization

Drug entrapment efficiency

The drug loading (drug incorporated onto the 1 mg functionalized MWNTs out of 4mg initially taken) were determined by passing the 1ml formulation through Sephadex G-50 column, washing the column with 1 ml phosphate buffer pH 7.4 and collecting the 4 fractions of 0.5 ml. Dilute the fractions with phosphate buffer pH 7.4 and measure the absorbance at 259nm using phosphate buffer pH 7.4 as blank. Calculate the drug loading and % assay (Kim et al., 1994).

Morphology

Particle size distribution study and zeta potential of the MWCNT conjugate was measured using Zetasizer Nano ZS (Malvern instruments, U.K.). TEM study of the MWCNT conjugate was conducted using Tecnai 20 Transmission electron microscope, Philips, Holland.

In-vitro drug release study (Wang et al., 2007)

The *in vitro* release study of Methotrexate from the MWCNTs formulation was determined using dialysis membrane. Briefly, 1 ml of MWCNTs formulation was taken in a dialysis tube (Mol. Wt. cut-off 12 000; HIMEDIA, Mumbai, India Himedia) and was suspended in phosphate buffer at a specified pH. Drug release from the formulation was determined by estimating drug content in the samples withdrawn at convenient intervals of time for 48 hours (Tekade et al., 2008).

Stability study

Stability study of MWCNTs formulation was carried out at room temperature (R.T.) and at refrigerated conditions (Freeze) for 1 month. Drug assay (%) and particle size were determined for samples withdrawn at specified time interval.

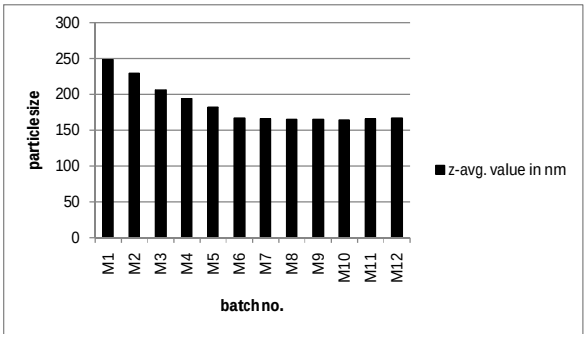

Fig.1 Optimization of concentration of DSPE-mPEG.

RESULTS

Functionalization of Multi-Walled Carbon Nanotubes and drug loading

Different batches of functionalized MWCNTs were prepared for optimization of MWCNT: DSPE-mPEG ratio by keeping concentration of MWCNT constant (1 mg/ml) & using varied concentrations of DSPE-mPEG as shown in Table 1.

Table. 1. Optimization of concentration of DSPE-mPEG in the formulation

Batch No.	Conc. Of DSPE-mPEG in mg/ml	z-average value in nm
M1	1	248.7
M2	2	229.8
M3	3	206.7
M4	4	194.5
M5	5	181.6
M6	6	166.5
M7	7	166.1
M8	8	165.2
M9	9	165.3
M10	10	164.0
M11	11	165.7
M12	12	166.9

Particle size has been taken as the response parameter for the optimization. The optimized batch (Batch M8) was prepared under varying conditions of processing to study the influence of sonication time on the particle size and drug entrapment respectively. Table 2 lists out the results obtained from such a study in the preparation of MWCNT-mPEG formulation.

Table. 2. Optimization of sonication time on the particle size of MWCNT-mPEG formulation.

Batch No.	Sonication time in min	Particle size (z-avg. value) in nm
M8a	15	235.7
M8b	30	220.9
M8c	45	210.2
M8d	60	196.4
M8e	75	178.9
M8f	90	165.2
M8g	105	164.6
M8h	120	162.4

Out of the above, based on the particle size, figure 2 explains 90 minutes was an optimum sonication time (Batch M8f). Similarly, experiments were conducted to optimize the concentration of MTX for maximum drug entrapment. As shown in Table 3, the concentration of MTX was increased from 2mg/ml to12 mg/ml keeping the functionalized MWCNT concentration constant (1mg/ml). Based on the % drug entrapment values, batch M16 was optimized.

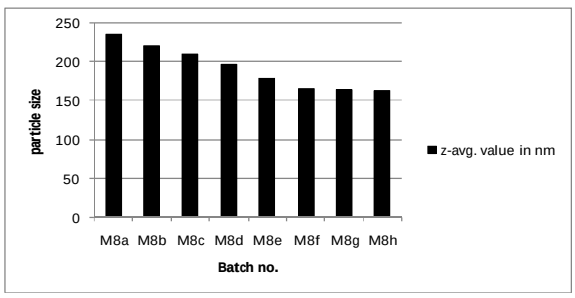

Fig.2 Optimization of sonication time.

The optimized batch (M16) shown in figure 3 was prepared under varying conditions of processing to study the influence of incubation period on the drug entrapment. Table 4 lists out the results obtained from such a study in the preparation of MTX loaded MWCNT-mPEG formulation.

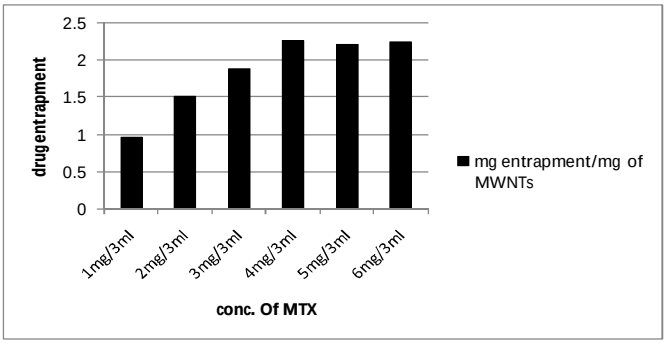

Fig.3 Optimization of drug entrapment efficiency.

Table. 3. Optimization of drug entrapment efficiency

Batch No.	Conc. Of MTX	z-avg. value in nm	Entrapment/ 1mg MWNTs	% Entrapment
M13	1mg/3ml	189.0	0.96mg	96.0%
M14	2mg/3ml	192.0	1.51 mg	75.5%
M15	3mg/3ml	186.5	1.89mg	63.0%
M16	4mg/3ml	185.2	2.26mg	56.5%
M17	5mg/3ml	197.0	2.21mg	44.2%
M18	6mg/3ml	196.9	2.25mg	37.5%

Tab. 4. Optimization of incubation time on percent drug entrapment.

Batch No.	Incubation period	z-avg. value in nm	Entrapment/ 1 mg MWNTs	% entrapment
M16a	12 hr	182.2	2.08 mg	52.0%
M16b	24 hr	185.1	2.26 mg	56.5%
M16c	36 hr	185.2	2.26 mg	56.5%
M16d	48 hr	187.9	2.27 mg	56.75%

Characterization of formulation

The optimized batch (M16b) of MTX loaded DSPE-mPEG formulations were characterized for the following parameters;

1) Particle size analysis
2) Zeta potential
3) Transmission electron microscopy (TEM)

Particle size analysis and zeta potential measurement:

The final freeze dried formulation of Methotrexate loaded PEGylated carbon nanotubes showed a mean particle size of 189.7 nm with 0.215 PDI and 100% peak intensity when the formulation was reconstituted using pyrogen free water (Figure 4). The Zeta potential of the same formulation was found to be -25.8 mV (Figure 5).

Transimission electron microscopy (TEM)

TEM study shows the internal structure of functionalized multi walled carbon nanotubes (Figure 6A) including the external and internal tube diameter. The internal diameter of the MWNTs is about 7.36 nm and the wall thickness is 4.29 nm (Figure 6B). Figure 6C shows length of MWNTs i.e. about 191.7nm. The

particle size correlates with that obtained from the z-avg. data obtained from measurement by Zetasizer.

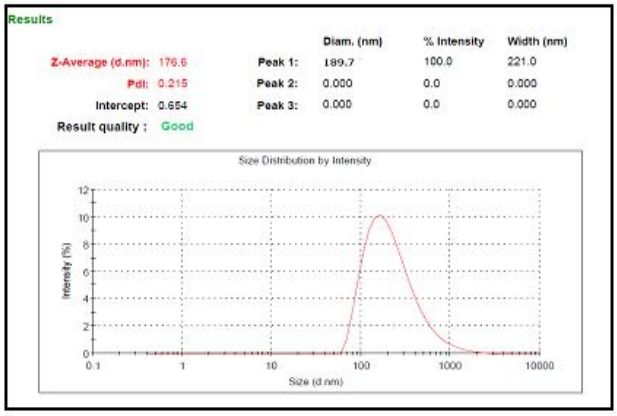

Fig.4. Particle size of formulation

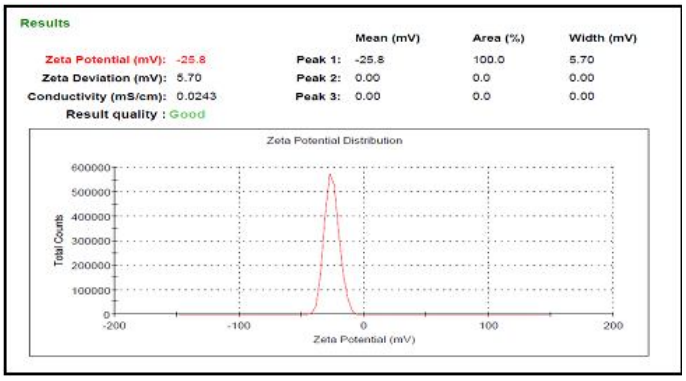

Fig. 5. Zeta potential of formulation

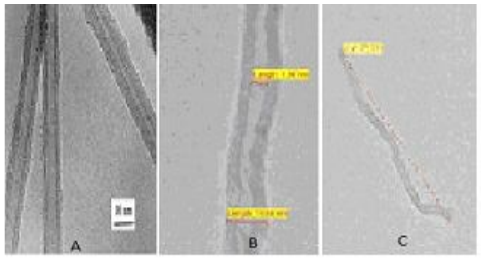

Fig. 6. TEM images of MWCNTs showing (A) the internal and external surface structure, (B) internal, external diameter and (C) length measured on a linear scale.

In-vitro drug release study

The MTX release profile from the MTX loaded MWCNT-mPEG formulation in phosphate buffer of pH 7.4 and pH 5.8 were compared as given in Figure 7. The drug release from both the plain drug and MTX loaded MWCNT-mPEG formulation were linear but the rate was substantially high in pH 5.8 phosphate buffer (58.2 %) while, the release was slow in pH 7.4 phosphate buffer (44.2 %) in case of MTX loaded MWCNT-mPEG formulation in 28 hours. Virtually, no difference in the release rate with change in pH was observed in case of plain MTX. The release was comparatively rapid up to 8 hours followed by slow release up to the end of the study period. The overall drug release was only 58.2 % from MTX loaded MWCNT-mPEG formulation at 5.8 pH.

Stability study of formulation

Stability study was conducted at room temperature and at refrigerated condition to study the influence of storage on the drug assay and particle size. The details of results are represented as bar graph in Figure 8a and Figure 8b with respect of particle size and drug assay respectively. The results showed that the formulations were more stable during the period of study (5 weeks) at refrigerated conditions.

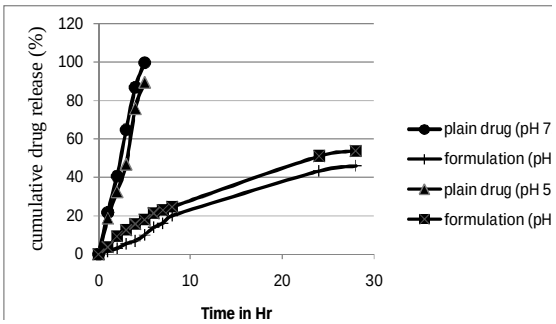

Fig. 7. In-vitro drug release profile in pH 5.8 and pH 7.4 phosphate buffer of MTX plain drug (triangle & circle) and MTX loaded MWCNT-DSPE-mPEG formulation (square & plus).

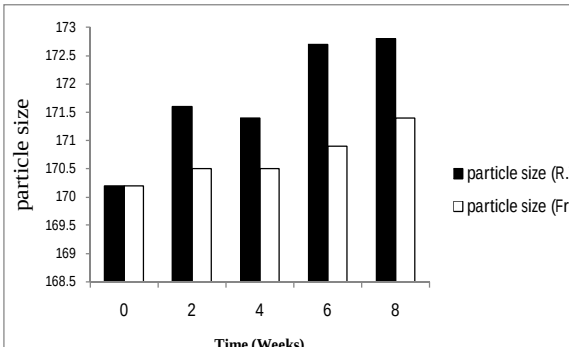

Fig.8a Stability study: influence of storage on particle size.

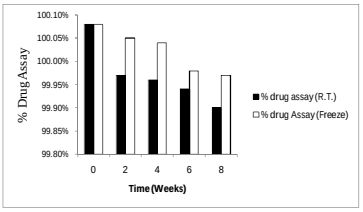

Fig. 8b Stability study: Influence of storage on % drug assay.

DISCUSSION

Among drug delivery systems, MWCNT's are able to enhance delivery, absorption and intracellular uptake of a bioactive molecule while protecting it from deactivation, Carbon Nanotubes (CNTs) have emerged as a recent and promising option especially in cancer therapy. This is mainly due to their unique properties,

which render them extremely versatile through the incorporation of several functional groups and targeting molecules at the same time, while their natural shape allows them to selectively penetrate across biological barriers in a non-invasive way (Pastorin, 2009). As such, CNTs are highly hydrophobic and not dispersible in aqueous systems and hence needs functionalization.

Functionalization of CNT involves grafting of chemical functions at the surface of the nanotubes by covalent or non covalent linkage to add new properties for CNTs. In the present study, Non-covalent functionalization of Multi-Walled Carbon Nanotubes (MWNTs) was conducted using DSPE-mPEG to impart aqueous dispersion property to the carbon nanotubes. Functionalization was achieved by incubating carbon nanotubes with DSPE-mPEG in the ratio of 1:8 because figure 1 showed constant z-avg. value still addition of DSPE-mPEG, which yielded the product with 165.2 nm z-avg. The particles were found to have visibly good dispersion property after functuionalization. This extra-ordinary character of dispersion is due to the formation of complexes formed due to the irreversible adsorption of PEG molecules to the sidewalls of carbon nanotubes through π-stacking, van der Waals and hydrophobic interactions (Chen et al., 2003). Report by Z. Liu et.al., 2008 also confirms that noncovalently functionalized carbon nanotubes by PEGylated phospholipids were stable without aggregation in various biological solutions including serum (Liu et al., 2007, Kam et al., 2005). Poly (ethylene glycol) (PEG) functionalization allows nanotubes for surprisingly high degrees of π-stacking of aromatic molecules, including a cancer drug and combinations of molecules. Here, figure 3 showed that Methotrexate was loaded onto the functionalized MWCNTs with reasonably good percentage of entrapment (2.26 mg of methotrexate per mg of carbon nanotubes with 56.5% entrapment efficiency). An increase in the particle size was noted (185.2 nm z-avg.) after incubation of the functionalized MWCNTs with methotrexate solution in phosphate buffer pH 7.4 for 24 hours. However, the size is well with in the range that favors long blood circulation because the average length exceeds the threshold for renal clearance (Soo Choi et al., 2007). Even though increase in incubation time up to 24 hours increased the percent entrapment, further increase in incubation time failed to improve entrapment any more. (Table 4). In an attempt to increase the entrapment efficiency, fixed concentration of the functionalized MWCNTs were incubated with fixed volumes of MTX solution of varied concentration ranging from 1mg/3ml to 6 mg/3ml. A progressive decrease in % entrapment was observed with increase in the concentration of MTX (Table 3). However, calculating the amount of MTX conjugated per mg of MWCNT, it is evident that batch M16 showed maximum drug entrapment on w/w basis (2.26 mg MTX per mg of MWCNT) (Figure 3). Hence this batch was selected for future investigations. The Zeta potential of the same formulation was found to be negative (-25.8 mV) at physiological pH (Phosphate buffer).

TEM study showed the external and internal tube diameter to be 7.36 nm and 16.00 nm respectively with the wall thickness of about 4.3 nm and the length of about 191.7nm calculated on the linear basis. The overall thicknesses of the nanotubes were found uniform throughout the length. The TEM images (Figure 6) also show nearly smooth internal wall but the outer wall was rough with slight bulging at various points. This indicates the possibility of functionalization and conjugation of DSPE-mPEG and MTX on the outer surface of the carbon nanotubes. A similar result was reported by H. Kong et.al., 2005 with two kinds of polyelectrolyte: polyacrylic acid (PAA) and poly (sodium 4-styrenesulfonate) (PSS), where the grafted polymers were anchored onto the convex surfaces of multiwalled carbon nanotubes (MWNTs).

The release of MTX from MTX loaded MWCNT-mPEG formulation in to phosphate buffer of pH 7.4 and pH 5.8 was studied and results were compared with the plain drug (Figure 7). The drug release from all the formulation were linear but in case of MTX loaded MWCNT-mPEG formulation, the rate was substantially high in pH 5.8 phosphate buffer than in pH 7.4 phosphate buffer. This effect is because of the fact that the strength of π-stacking of aromatic molecules is dependent on nanotube diameter and pH of the medium and so this parameter has been effectively used as a method for controlling the release rate of molecules from nanotube materials (Liu et al., 2007). Obviously, cancer tissue matrix being acidic, the formulation can expect to release into the cancer cells as compared to other parts of the body more effectively. In comparison to plain drug, the release rate of MTX was slow from both the formulations.

Stability study of MWCNT-mPEG-MTX formulation conducted at room temperature and at refrigerated condition showed that the formulations were stable during period of study (5 weeks). Under both conditions, no significant change in the drug content and particle size was observed but a significant change in the release rate was recorded. Overall, the stability was higher under refrigerated condition.

CONCLUSION

Methotrexate loaded PEGylated carbon nanotubes were prepared by functionalization of MWCNTs effectively with drug loading capacity of about 2.26 mgs of MTX per mg of MWCNT. The formulation showed higher drug release at acidic pH rendering the formulation suitable for cancer delivery. Further study can be carry out to show low RES uptake of functionalized MWCNT and making the formulation a long circulating, while possibly reducing liver toxicity. MTX loaded PEG functionalized MWCNT can be showed significant uptake by the lungs when tested in mice in future.

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