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MANNOSYLATED MULTIWALLED CARBON NANOTUBES ASSISTED ARTESUNATE DELIVERY FOR CEREBRAL MALARIA

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ABSTRACT

Objective: The present investigation focused on the novel approach using artesunate (AS) loaded mannosylated conjugated multi-walled carbon nanotubes (M-MWCNTs) for site-specific delivery to the brain in the treatment of cerebral malaria (CM).

Methods: The raw MWCNTs were purified by selective oxidation method and then exposed to sequential chemical functionalization according to the following steps: carboxylation, acylation, amine modification and finally, D-mannose conjugation. The AS was loaded via the equilibrium dialysis method in the molar ratio 1:3 of various functionalized sonicated MWCNTs. The functionalized MWCNTs were characterized for elemental analysis, FTIR, TEM, zeta potential and percentage drug entrapment efficiency. The *in vitro* drug release study was performed on AS conjugated purified MWCNTs (AS-P-MWCNT) and AS conjugated M-MWCNTs. Bio-distribution study was performed on albino rat for quantitative measurement of AS in different organs and blood.

Results: The TEM images of M-MWCNTs indicated their open tubular nature and AS-M-MWCNTs suggests the entrapment of AS. The percent drug entrapment of AS-M-MWCNT was found to be 80.29±3.4 %. *In vitro* AS release from AS-M-MWCNTs was found in a controlled manner at pH 7.4. The bio-distribution studies clearly indicate the superiority of the AS-M-MWCNTs, as compared to the plain drug towards increasing the accumulation of AS in brain.

Conclusion: The results suggest that AS-M-MWCNTs could be employed as an efficient nano-carrier for antimalarial therapy in cerebral malaria.

Keywords: Carbon Nanotubes, Cerebral malaria, Artesunate

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INTRODUCTION

Malaria is a protozoal disease in humans elicited by protozoan parasites transmitted through female species of Anopheles mosquito of genus *Plasmodium*. Among five types of parasites plasmodium falciparum is the most virulent and cause serious complication of the infection known as cerebral malaria (CM). The principal mechanism leading to CM is adherence (mediated by many ligands and receptors) and sequestration of parasitized erythrocytes and immune cells to endothelial cells presents in brain capillaries via induction of an inflammatory process and imbalance in the production of neurotoxic and neuroprotective factors which lead to inflammation of the brain and/or death of humans in severe cases [1, 2].

For the management of CM several drugs like quinine or artemisinin [3], are currently used routinely with conventional drug delivery system. The main drawback of conventional malaria chemotherapy is non-specific targeting to intracellular parasites, resulting in higher dose requirements, subsequent intolerable toxicity and the development of multiple drug resistance against parasite. Therefore, an urgent requirement for designing novel drug targets or compounds against this parasite is needed for its management. Formulation and evaluation of novel drug delivery systems is not only less expensive than developing new drugs but may also improve the delivery of existing anti-malarial drugs at the desired rate. Artemisinin derivative has many advantages over other antimalarial drugs, among them commonly used semi-synthetic derivative Artesunate (AS, $C_{19}H_{28}O_8$) is mainly useful for the multidrug-resistant case of falciparum malaria or Cerebral Malaria (CM). Artemisinin is a sesquiterpene lactone isolated from the plant Artemisia annua and known for its ability to denature the number of *plasmodium* parasites in the bloodstream and deep organs including the brain by the coagulation of protein present in parasite DNA. It acts on the trophozoite stage of the life cycle of the malaria parasite and stops the formation of schizont.

Carbon nanotubes (CNTs) have established much recent interest as new entities as a novel drug delivery system for experimental disease diagnosis and treatment because of their unique properties to provide a hollow core appropriate for storing guest molecules [4, 5]. Important properties of CNTs, making them a famous tool more than other nanocarriers, are greater stability, biocompatibility, non-immunogenicity, ease of size alteration and high drug-loading potential [6]. Internal and external surfaces of CNTs can be modified on an individual basis as required and a variety of functional groups can be generated on their surface in support of further conjugation with targeting ligands as well as drug molecules. The CNTs can be also degraded within human brain tissue by myeloperoxidase (MPO) and hydrogen peroxide (H_2O_2) [7].

The present investigation focuses on the novel approach of using multi-walled carbon nanotubes, which are purified and then surface modified by mannose to target the brain cells having the mannose receptors (transmembrane glycoprotein) expressed by macrophages and binds to mannosylated molecule and mediated their endocytosis to deliver the anti-malarial moiety at desired site and treat the disease in an effective manner. Hence, we are reporting for the first time that mannose conjugated multi-walled carbon nanotubes loaded with anti-malarial drug artesunate to target brain cell serve as a good inhibitor of *plasmodium falciparum* and can act as a controlled release system.

MATERIALS AND METHODS

Materials

Multiwalled carbon nanotubes (MWCNTs) (carbon basis>90%) having diameter x length, i.e. 110-170 nm x 5-9 µm, D-mannose and PTFE filters (0.45 µm pore size) were purchased from Sigma-Aldrich, USA. Artesunate was obtained as a gift sample from Solisto Pharma Sagar, M. P. INDIA. Dialysis membrane (MWCO, 12–14 KDa) was purchased from Himedia Laboratories Pvt. Ltd. (India). All other

chemicals and reagents were of analytical grade and deionized water has been used for all experiments.

Purification (of raw MWCNTs)

By minor modifications the MWCNTs were purified by selective oxidation method [8]. Purification techniques basically, divided into two main streams: Structure selective (to separate the CNTs from the impurities) and size selective separations (provide more homogeneous diameter or size distribution).

Oven purification

The R-MWCNTs were treated at 250±2 ℃ for 1 hr in hot air oven (Joyti Scientific Industries, India) to get rid of the metallic impurities and amorphous carbon because of elevated temperature to obtain P-MWCNTs.

Acid purification

After the heat treatment, the P-MWNTs (500 mg) were reacted with concentrated hydrochloric acid under magnetic agitation (Remi, Mumbai, India) for 5 hr, and filtered with 0.45 µm polytetra-fluoroethylene (PTFE) filter. It was used for the elimination of catalytic and amorphous impurities from the unpurified Raw-MWCNTs to get P-MWCNTs [9, 10].

Chemical functionalization

The P-MWCNTs were subjected to sequential chemical functionalization steps according to the following steps: carboxylation, acylation, amine modification, and mannosylation.

Cutting and carboxylation of MWCNTs

Carboxylation (cutting and oxidation) of P-MWCNTs were performed to generate carboxyl (-COOH) groups onto the surface and sidewall of MWCNTs. After the heat treatment, the purified MWNTs were refluxed with the acid mixture, i.e. sulphuric acid (98%) and nitric acid (68%) (H_2SO_4: HNO_3:: 3:1 v/v) at 60±2˚C for 4 to 6 h followed by washing through deionized water by centrifugation until the pH became neutral and filtered by 0.45 µm PTFE filters (Hangzhou Anow Microfiltration Co. Ltd., China). The acid treatment was performed under two different reaction conditions- at varying temperatures (25 °C, 60 °C, 80 °C, 100 °C) and for different time durations (1, 2, 4 and 6 hr) (tables 1 and 2). After that C-MWCNTs were dried at 100 °C in a vacuum oven (Jyoti Scientific Industries, Gwalior, India) to remove carbon dioxide and water. During this process, the mixed acid not only grafted functional groups onto the surface of the MWNTs but also dissolved metal particulate impurities. C-MWCNTs (solid) were transferred into a sanction tube (Soniweld, Mumbai) containing water and sonicated for 15 min to reduce the size of MWCNTs. The percentage yield was calculated by weighing the MWCNTs obtained after drying. For the determination of the acidic sites concentration present on C-MWCNTs were added into unreacted NaOH was titrated with 0.01 N hydrochloric acid (HCl) to each sample of carboxylated MWCNTs [11, 12].

Acylation of carboxylated MWCNTs

At 50 °C, 300 mg of C-MWCNTs were stirred in 63 ml mixture of thionyl chloride and dimethylformamide ($SOCl_2$: DMF: : 60:3) for 3 d to get (-CoCl) group on the tubes, followed by washing with anhydrous tetrahydrofuran (THF) by centrifuged at 8000 rpm for 20 min and about 5-6 times for the elimination of excess thionyl chloride. The black remainder of Acy-MWCNTs was fully dried and characterized [8, 13].

Amine modification of Acy-**MWCNTs**

Half (150 mg) of Acy-MWCNTs were reacted with the 70 ml of ethylenediamine (EDA) solution for about 4 d at 100 °C to generate–NH_2 group onto the wall of MWCNTs, followed by cooling at room temperature then washed 5-8 times by ethanol to get rid of the excess diamine from the Am-MWCNTs by repetitive centrifugation. Remainder was dried overnight and characterized [13].

Preparation of mannosylated multiwalled carbon nanotubes

D-mannose (200 mg) was dissolved in 100 ml sodium acetate buffer (pH 4.0), followed by heating the mixture at 60±0.5 ℃ for an hour and continuously stirred in a magnetic stirrer (Remi, Mumbai, India) at ambient temperature for 72 h to ensure the completion of ring opening reaction. From this, 30 mg mannose was added to 10 mg of Am-MWCNTs and the mixture was stirred for 3 d to get the M-MWCNT's. The schematics presentation of mannosylation process is shown in fig. 1. Purification of M-MWCNTs was done by a dialysis membrane (MWCO 12–14 KDa) against deionized water for 12 h to remove detached mannose and other impurities. The volume remained in the dialysis bag centrifuged for 25 min and dried overnight in a vacuum oven. The characterization of M-MWCNTs was done by FTIR spectroscopy (Perkin-Elmer, USA). Quantitative ninhydrin Kaiser Test was used to determine the degree of mannosylation in M-MWCNTs. The aqueous dispersion of MWCNTs was mixed with two drops of Kaiser A (0.5 ml of 0.065% w/v aqueous KCN solution, with 24.5 ml of dry pyridine and 2.5 ml of 400% w/v phenol/ethanol mixture) and Kaiser B (5%, w/v, ethanolic ninhydrin solution) in a microcentrifuge after that heated on a preheated block for 15–30s. The resultant was filtered (Milipore, 0.45 mm) and estimated spectrophotometrically (UV-1601, Shimadzu, Japan) [10, 14].

Characterization of functionalized MWCNTs

Elemental analysis

Elemental analysis of various functionalized MWCNTs were conducted to determine the S percentage composition in a Flash EA 112 series (Thermo Electron Corp., Italy) (table 3).

Fourier transform infra-red (FTIR) spectroscopy

FTIR spectroscopy was performed for each step during functionalization to confirm the presence of functional group on MWCNTs. FTIR spectrum were recorded over a scan range of 400–4000 cm−1 (Perkin-Elmer, USA) (fig. 2).

Morphology of MWCNTs and functionalized MWCNTs

Transmission electron microscopy

The size and shape of pristine and functionalized MWCNTs samples were characterized by Transmission Electron Microscopy (TEM) after drying on a copper grid and staining negatively by 1% phosphotungstic acid (PTA) by the metal shadowing technique [Morgani 268-D, Holland; fig. 3(a-d)].

Zeta potential

The zeta potential of purified and various functionalized MWCNTs were determined by Malvern Zeta sizer (Malvern Instrument, UK). The zeta potential of raw, purified and various functionalized MWCNTs was determined with 0.05 mg/ml concentration of MWCNTs suspended in double deionized water (pH 7.0) (table 4).

Drug entrapment efficiency of Artesunate in functionalized MWCNTs

The equilibrium dialysis method was used for the loading of drug AS into the P-MWCNTs. Known molar ratio 1:3 of different functionalized sonicated MWCNTs and AS were taken with PBS (pH 7.4) and incubated for 24 h with continuous magnetic stirring at 50 rpm (Remi, Mumbai, India). The total mixture was then dialyzed using a dialysis bag (MWCO 12-14 KDa) against 50% ethanol in PBS (pH 7.4) under the sink condition for 30 min to remove unentrapped AS. Amount of entrapped drug was determined indirectly by estimating the amount of free AS at 209 nm using UV-visible spectrophotometer (Shimadzu-1800, Japan). The formulation of dialysis bag was filtered, lyophilized (Hetro Dry Winner, Germany) and used for further characterization. The same method was followed for the loading of AS in functionalized-MWCNTs. The percentage entrapment of drug in different formulations was calculated using an equation: [15].

$$\textbf{\%}\ \text{Entrapment Efficiency} = \frac{(\text{Weight of entraped AS})}{(\text{Weight of entraped AS} + \text{Free AS})} \times 100$$

***In vitro* release studies**

The *in vitro* release of AS from the drug-loaded P-MWCNTs (AS-P-MWCNTs) and AS loaded mannosylated MWCNTs (AS-M-MWCNTs)

was evaluated in PBS, pH 7.4 by equilibrium dialysis tube diffusion technique (MWCO 12–14 KDa; Himedia, India). Briefly, the drug loaded replace by P-MWCNTs and M-MWCNTs. MWCNTs equivalent to 5 mg of the AS was taken in a dialysis tube and dialyzed against 50 ml of release medium at room temperature with constant stirring at 30 rpm on a magnetic stirrer (Remi, India). One ml of the sample was withdrawn at each scheduled time interval and replenished with an equal volume of the fresh medium to maintain the sink condition. The samples were appropriately diluted with the distilled water and were analyzed for AS using UV-visible spectroscopy at 209 nm. Results are shown in (fig. 4).

Tissue bio distribution study

Albino rats (Wistar strain) were weighed, marked and then divided into 4 groups of 9 each (n=3). Rats were fasted overnight and allowed access to water ad libitum before the experiment. Free AS solution (free AS), AS-P-MWCNTs and AS-M-MWCNTs containing an equivalent dose of AS (1.0 mg/kg body weight) were administered through IV route to rats of first, second and third groups, respectively. The fourth group of animals were kept as control**.** The animals from each group were sacrificed at 1, 6 and 24 h time points and the blood were collected via cardiac puncture in anti-clot vials (Himedia, India). Various organs viz. liver, spleen, kidney and brain were carefully excised, isolated, washed with normal saline, were blot dried and stored at-80 °C until assay. The tissue biodistribution levels of AS were monitored by UV spectroscopy method. The results were expressed as percentage drug distribution. The data of biodistribution from AS, AS-P-MWCNTs and AS-M-MWCNTs formulations are shown in (fig. 5). All investigations were performed as per the protocol approved by the Institutional Animals Ethical Committee of Adina Institute of Pharmaceutical Sciences, Sagar (M. P.) India.

Statistical analysis

Statistical test was performed with Graph Pad Instat Software (Version 3.00, Graph Pad Software) by one-way ANOVA followed by Tukey–Kramer test for multiple comparisons. A probability $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Preparation and characterization of mannosylated multi-walled carbon nanotubes

Generally, R-MWCNTs hold several impurities like metals and amorphous carbon in particulate form therefore, strong acid treatment and hot air oven are the two most commonly used techniques for the purification. This removes the amorphous carbon as well as metallic impurities. These impurities were detached to get P-MWCNT's by oxidative strong-acid treatment, metals were removed by forming metal oxides and confirmed by elemental analysis.

The strong acid treatment also enhances the nanotubes solubility in aqueous media, separates impurities and shortened MWCNTs [13, 16]. In performing acid treatment of the MWCNTs it was observed that with increasing temperature and time, the length of MWCNTs decreases, hence the time and temperature where optimum length received were found to be 4 h and 80 °C, respectively (table 1 and 2). The purified MWCNTs were modified to generate more functional groups and then carboxylated by treating with an acidic mixture of sulfuric acid (98%) and nitric acid (68%) to generate carboxyl (-COOH) groups onto the surface and sidewall of nanotubes. The quantification of the number of acidic groups and their corresponding length was determined by acid-base titration and TEM. The C-MWCNT'S were then acylated using thionyl chloride and dimethyl-formamide (DMF) and washed with the anhydrous tetrahydrofuran (THF) for the removal of excess thionyl chloride, for about 5-6 times to produce (-CoCl) groups, and processed to get amine modified MWCNTs by using ethylenediamine; EDA to generate ($-NH_2$) group on the surface of the tubes and were washed with ethanol 5-8 times to remove the excess diamine solution by well-known reported methods [12]. The number of free amino groups as determined by quantitative Ninhydrin Kaiser test was found to be 3.1 mmol/g of amine-modified MWCNTs.

Table 1: Temperature variation for carboxylation of MWCNTs at 1 h

Temperature (°C)	-COOH concentration mmol/g	Length (nm)
25	1.4±0.24	920±28
60	2.1±0.36	815±22
80	**3.5±0.45**	**720±24**
100	3.6±0.53	721±26

For mixture of H_2SO_4/HNO_3 (3:1). Values represent mean±SD (n = 3).

Table 2: Time variation for carboxylation of MWCNTs at fixed temperature (80 °C)

Time (h)	-COOH concentration mmol/g	Length (nm)
1	3.5±0.45	720±26
2	7.1±0.68	450±10
4	8.5±0.59	210±21
6	8.6±0.62	215±22

For mixture of H_2SO_4/HNO_3 (3:1). Values represent mean±SD (n = 3).

Then, mannosylation was carried out by ring opening of mannose followed by reaction of its aldehyde groups with the free Am-MWCNTs in 0.1 M sodium acetate buffer (pH 4.0) [17]. This leads to the formation of Schiff's base (-N=CH-), which may then get reduced to secondary amines ($-NH-CH_2-$) but stay in equilibrium with Schiff's base. Free mannose and impurities were isolated by dialysis and characterized for the presence of a mannose residue by ligand agglutination assay, i.e. Concanavalin A assay. The mannose conjugation to MWCNTS is schematically shown in fig. 1.

Various treated MWCNTs were heated at 55 °C to remove carbon dioxide and water. MWCNTs were transferred into a sonication tube (Soniweld, Mumbai) containing water and sonicated for 15 min (for size reduction). The different functionalized MWCNTs were then subjected to characterization to revel various parameters.

Mannosylation was carried out by coupling amine group present on the surface of MWCNTs. Broad, intense O-H stretch and C-O stretch of mannose around 3400.22 cm^{-1} and 1641.85 cm^{-1} respectively, and N-H deformation of secondary amine at 1565.87 cm^{-1} confirmed the Schiff's base formation and some amine formation in the linkage between an aldehyde of mannose and amine termination groups of multi-walled nanotubes.

Elemental analysis of raw MWCNTs, purified, carboxylated, acylated, amine modified and Mannosylated MWCNTs was performed to determine content purity. The carbon content was found to be 97.36% and 99.12% for R-MWCNTs and P-MWCNTs respectively, which clearly indicates that no other element was present in P-MWCNTs that would affect the integrity and properties of MWCNTs. The increased hydrogen percentage in the case of C-MWCNTs clearly

suggests that some carboxyl groups have been attached during carboxylation process. The increased nitrogen percentage in the case of Am-MWCNTs clearly suggests that some amine groups have been attached during amine modification of MWCNTs. M-MWCNTs depict increased percentage of hydrogen clearly suggesting attachment of mannose on the carbon surface. Elemental analysis of the MWCNTs exhibiting the percentage of carbon, hydrogen and nitrogen shown in the table 3.

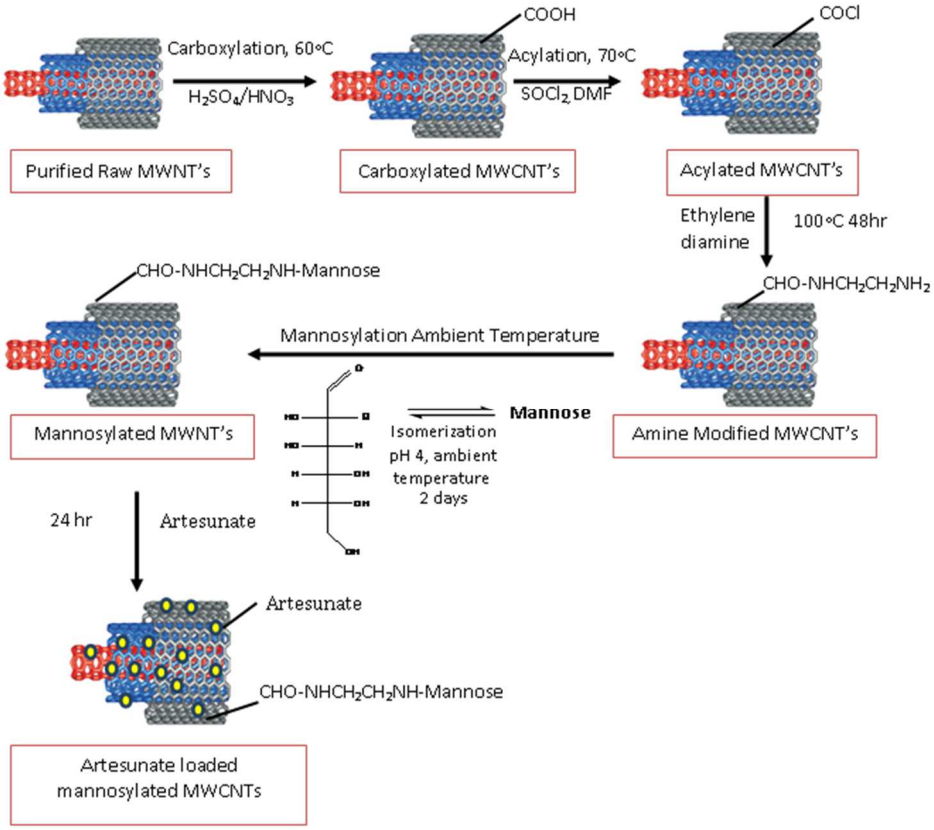

Fig. 1: Schematic representation of MWCNTs functionalization

Table 3: Elemental analysis of different functionalized MWCNTs

Sample	Atomic percentage±SD (%)		
	Carbon	Hydrogen	Nitrogen
Raw-MWCNTs	92.36±3.2	-	-
P-MWCNTs	99.12±3.7	-	-
C-MWCNTs	74.91±2.6	1.3±1.2	1.93
Acy-MWCNTs	70.45±3.1	1.2±1.1	1.90
A-MWCNTs	68.37±3.5	3.5±2.2	8.2±1.1
M-MWCNTs	62.39±3.1	6.2±1.3	5.9±1.2

Values represent mean±SD (n = 3).

Fourier transform infrared spectroscopy

FTIR study was performed on functionalized MWCNTs to assess the presence of various functional groups over their surface. The P-MWCNTs show less dense peaks at 3436.36 cm^{-1}, 2853.41 cm^{-1} and 1120.18 cm^{-1}, which could be ascribed to the O-H stretching, C-H stretching and O-H in-plane bending, respectively (fig. 2). Data confirms the presence of some oxygenated groups generated after the purification process. C-MWCNTs showed few broad, strong peaks at 3416.80 cm^{-1}, 2923.44 cm^{-1}, 1075.91 cm^{-1}, 1639.01 cm^{-1}, 1417.92 cm^{-1} and 1025.9 cm^{-1}, which could be ascribed to O-H stretching, C-H stretches, C=O stretching, C=C stretching, C-O is stretching and O-H bending, respectively (fig. 2). The data confirm the presence of carboxylic (-COOH) groups present on the surface of MWCNTs. As functionalization proceeds numbers of peaks increases due to the further attachment of other functional groups. FTIR analysis proved the formation of Schiff's base and secondary amine (-NH-CH_2-) linkage between aldehyde group of mannose and Am-MWCNTs. M-MWCNTs showed the peak at 1567.80 cm^{-1}, 1646.66 cm^{-1}, 2925.64 cm^{-1}, and 3401.53 cm^{-1}, which could be ascribed to–C=N stretching (Schiff 's base formation), C-H stretch of CH_2, O-H stretching respectively (fig. 2). AS-M-MWCNTs showed the peak at 1587.80 cm^{-1}, 1646.66 cm^{-1}, 1379.45 $cm^{-1}$and 2949.18 cm^{-1} which could be ascribed to–C=N stretching due to Shiff's base,-C=O stretch of amide bond, C=O bond, C-H stretch of CH_2 respectively (fig. 2).

Zeta potential

The R-MWCNTs shows positive zeta potential whereas P-MWCNTs shows a negative value of zeta potential (-44.5 mV), which could be due to the presence of carboxylic group during the purification process. Acy-MWCNTs showed negative value due to the negative inductive effect of chlorine. Similarly, the Am-MWCNTs shows a positive value due to protonation of the amino group. The M-MWCNTs shows comparatively lower, but still positive value of the zeta potential. The change in zeta potential strictly depends upon the pH of the medium (table 4).

Table 4: Zeta potential and drug entrapment efficiency of MWCNTs

Types of MWCNTs	Zeta potential (mV)	Percent entrapment efficiency of AS
Raw MWCNTs	+41.6±1.14	NA
P-MWCNTs	-44.5±1.86	54.35±2.23
C-MWCNT	-41.7±2.12	68.45±1.25
Acy-MWCNT	-17.2±0.76	62.22±1.36
Am-MWCNTs	+24.9±0.87	55.03±1.41
M-MWCNTs	+1.730±0.02	80.29±1.40

Values represent mean±SD (n = 3).

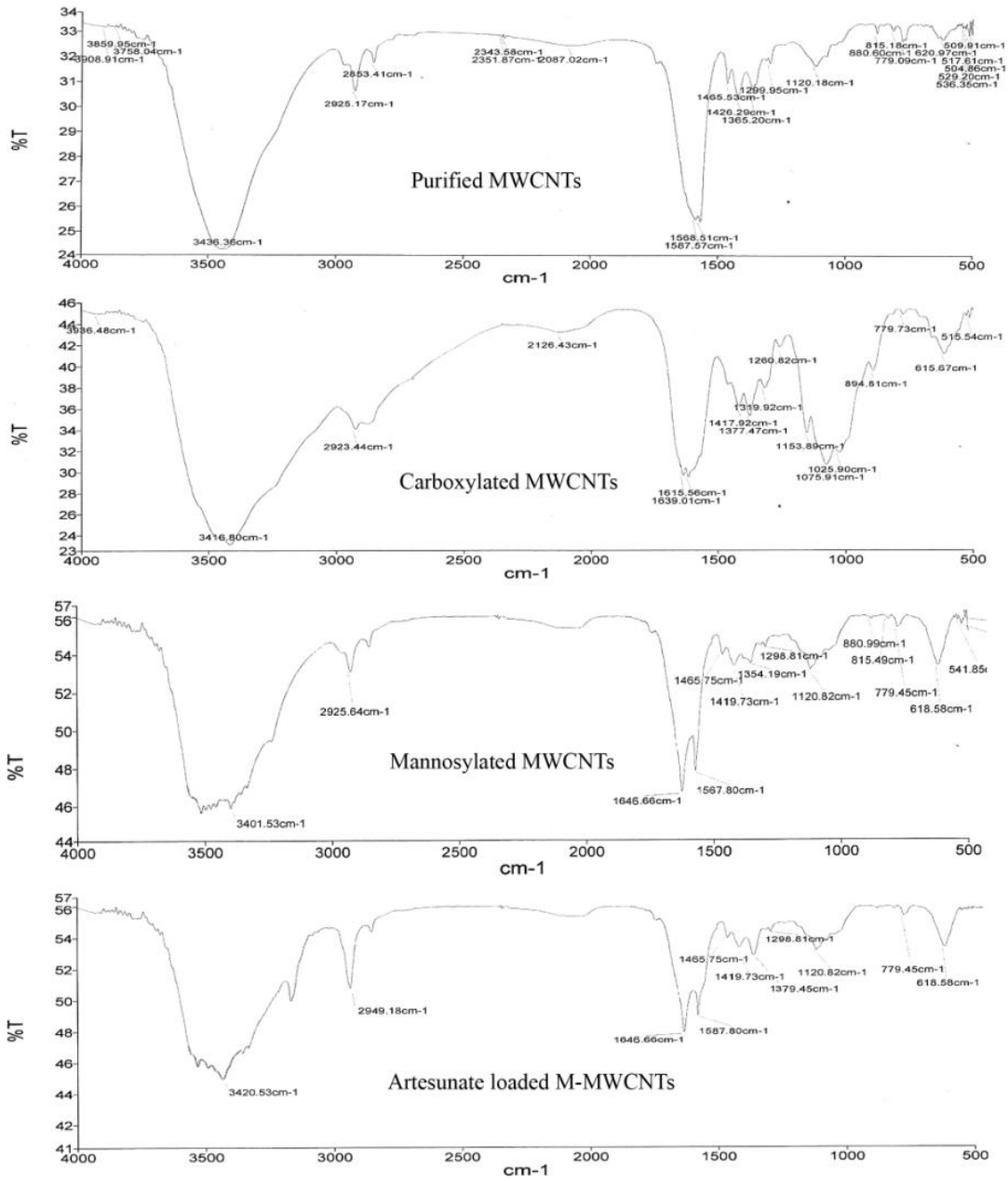

Fig. 2: FTIR spectrums of functionalized MWCNTs

Transmission electron microscopy

The lengths of R-MWCNTs, C-MWCNTs, M-MWCNTs and AS-M-MWCNTs were investigated by transmission electron microscope (TEM). C-MWCNTs showed a reduction of length of MWCNTs due to oxidation and formation of -COOH groups. During strong-acid treatment ends of MWCNTs, five-membered carbon rings and other defect sites result in the generation of -COOH group and cutting of MWCNTs. Hence, the TEM photographs showed the length of carboxylated and mannosylated MWCNTs to be around 500 nm as depicted in fig. 3 (b) and (c). The images of M-MWCNTs indicated their open tubular nature and AS-M-MWCNTs suggests the entrapment of AS (fig. 3).

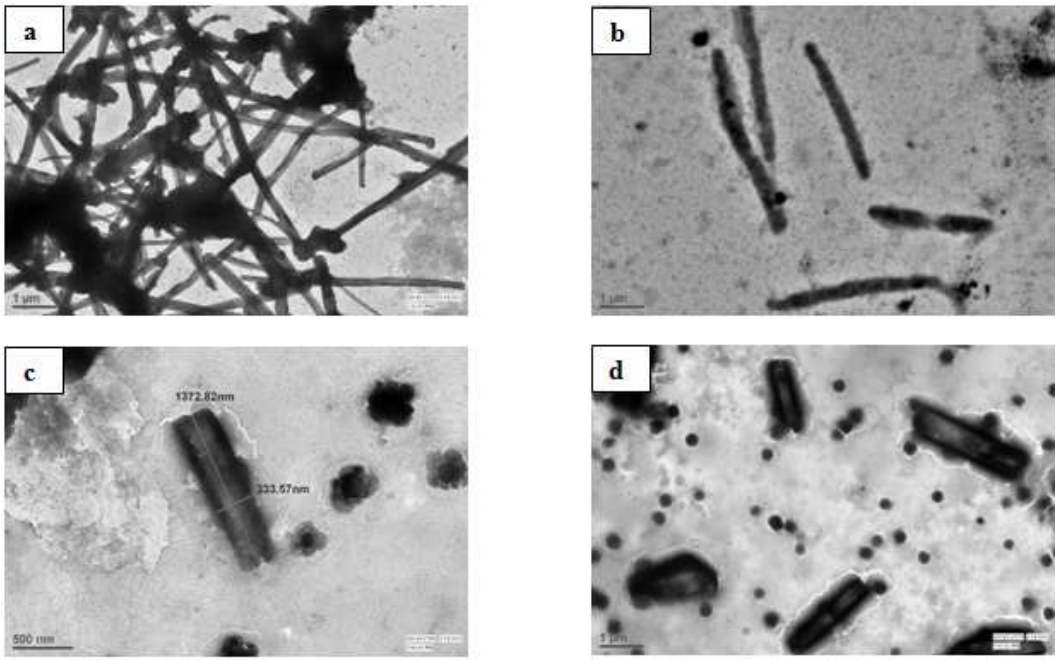

Fig. 3: TEM images of MWCNTs at (a) R-MWCNTs (b), C-MWCNTs and (c) M-MWCNTs (d) AS-M-MWCNTs

Percent drug entrapment efficiency

It was already reported that the drug can be easily adsorbed on sidewalls and surface of the CNTs (π–π stacking interactions) by mixing the drug with CNTs. Similarly AS molecules were entrapped in MWCNTs through π–π stacking as well as electrostatic interactions, which seem to be more stable in PBS [18]. Entrapment efficiency of AS in purified, carboxylated, acylated, amine modified and mannosylated MWCNTs was found to be 54.35±2.23, 68.45±1.25, 62.22±1.36, 55.03±1.41 and 80.29±1.40 respectively (table 4). The P-MWCNTs were present in the bundle state or in aggregates and very little surface area was available for the entrapment of drug in bundled MWCNTs which shows limited entrapment for AS-P-MWCNTs. However, C-MWCNTs showed a large amount of entrapped drug due to the opening of bundles of purified raw MWCNTs and presence of various pores generated by acid treatment which provides larger surface area and easy penetration into inner cavity of C-MWCNTs. While comparing the percent drug entrapment within different f-MWCNTs, the M-MWCNTs showed the highest percentage of AS entrapment and excellent dispersibility due to debundling.

***In vitro* release**

The *in vitro* release of AS from AS-P-MWCNTs and AS-M-MWCNTs was monitored under physiological conditions PBS (pH 7.4). The initial burst release achieved due to diffusion or the adsorbed AS on the surface of MWCNTs. It was observed that the release of AS was found to be sustained at PBS pH 7.4 and about 96.46% of AS was released in 100 h, possibly due to the presence of bulky mannose chain around the MWCNTs, which poses a hindrance to the drug release, compared to P-MWCNTs (fig. 4).

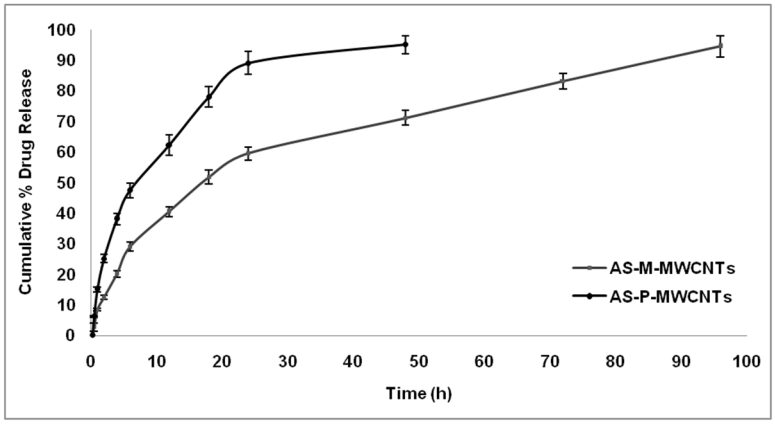

Fig. 4: Cumulative percentage drug release from AS-P-MWCNTs and AS-M-MWCNT's in PBS (pH 7.4) at 37 °C (n = 3). Each bar represents mean±SD (n = 3)

Tissue biodistribution study

A biodistribution study was performed for free AS solution, AS-P-MWCNTs and AS-M-MWCNTs formulations administered through IV route to compare the amount of AS that accumulates in various tissue like liver, brain, kidney and spleen at different time intervals. Amount of AS present in blood at various time intervals was also determined. One hr post IV administration of free AS solution (1 mg/kg), the

amount of drug present in blood was found to be very high along with accumulation in liver and spleen. AS was found to be rapidly cleared from blood within 24 h and the AS level in liver and spleen declined over the time and very low accumulation observed in the brain. In case of AS-M-MWCNTs increased level of AS (16.3±0.46%) accumulation observed in brain after 6 h and found even higher (22.4±0.78%) at 24 h, which may be due to receptor-mediated endocytosis. It was inferred that AS-M-MWCNTs were highly accumulated in brain and provided a sustained release of drug as compare to free AS and AS-P-MWCNTs. The biodistribution studies (fig. 5) clearly indicate the superiority of the AS-M-MWCNTs, as compared to the plain drug towards increasing the accumulation of AS in brain.

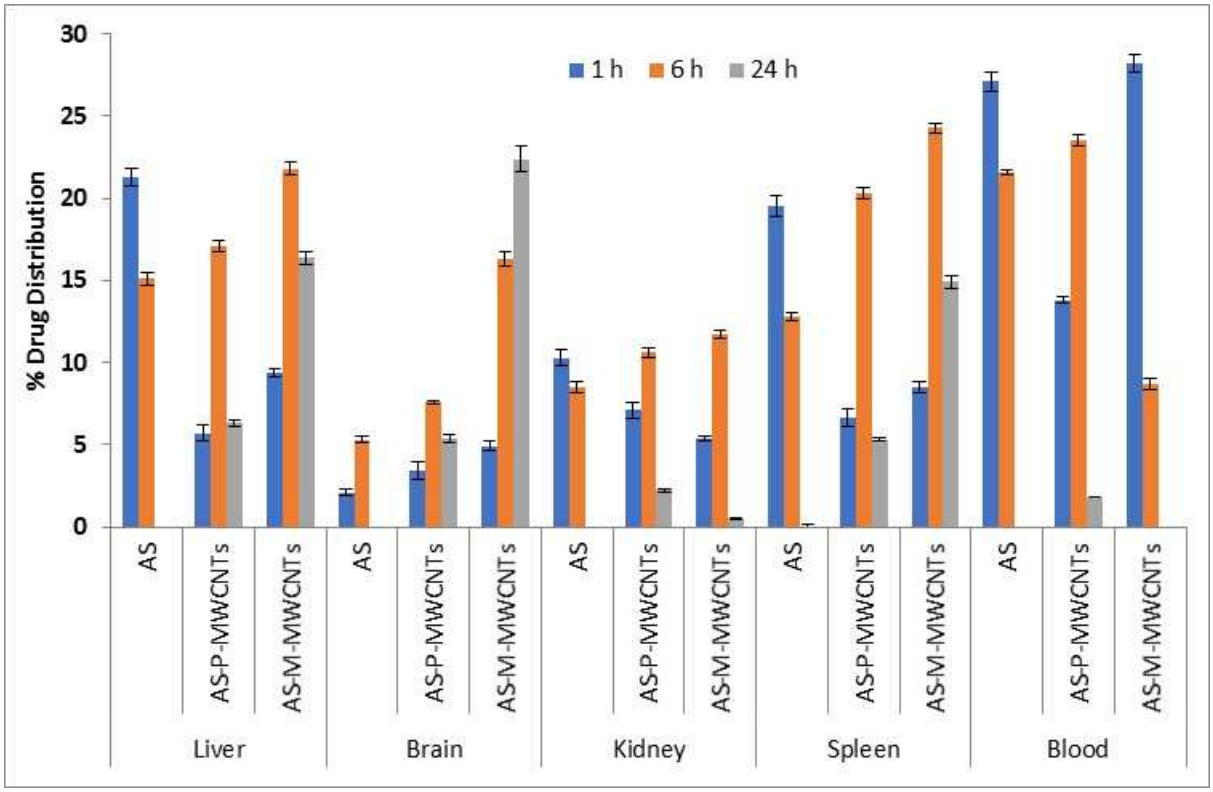

Fig. 5: Tissue biodistribution study of AS at 1h, 6h & 24h after i.v. injection of free AS, AS-P-MWCNTs and AS-M-MWCNTs in Albino rat.

CONCLUSION

The main problem in the treatment of CM is the ineffective amount of drug could reach to the brain due to lower shelf life in blood. In the present investigation AS loaded M-MWCNTs were developed to target the brain cells using the mannose receptor present at the membrane of various brain cells. From this study, it can be concluded that the AS loaded M-MWCNTs formulation showed efficient AS release to the desired site with an improved therapeutic margin of safety and an effective and alternative nano-formulation for the treatment of Cerebral Malaria in near future.

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ABBREVIATION

MWCNTs = Multiwalled carbon nanotubes, R-MWCNTs = Raw-MWCNTs, P-MWCNTs = Purified-MWCNTs, C-MWCNTs = Carboxylated MWCNTs, Acy-MWCNTs = Acylated MWCNTs, Am-MWCNTs = Amine Modified MWCNTs, M-MWCNTs = Mannosylated MWCNTs, AS-MWCNTs = Artesunate loaded MWCNTs, R-6G MWCNTs = Rhodamine-6G loaded MWCNTs, myeloperoxidase (MPO)

AUTHORS CONTRIBUTIONS

First, second and corresponding authors contributed equally. Third and fourth authors helped in data analysis and manuscript writing.

CONFLICTS OF INTERESTS

Declared none

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