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RATIONAL OF EMPLOYING CARBON NANOTUEBS IN ANTICANCER THERAPEUTICS

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ABSTRACT

Research on carbon nanotubes based anticancer drug delivery systems are gaining popularity because of various advantages over classical drug delivery systems including tumour targeting and improvement in the stability of drug molecules in the biological milieu. Nanotubes based anticancer formulations have shown a significant effect on cancer cell lines over various drugs currently available in the market. Due to their chemical structure and drug loading properties, they could be used as drug loaded cargo for delivery of anticancer agents. Moreover, their tumour targeting property is also been reported in hypoxic tumour cell lines where classical agents have failed to kill the cancer cells in a significant manner. This review focuses on various problems in anticancer drug delivery, ways and the rationale of tumour targeting by drugs, and structure of carbon nanotubes along with the methods of attachment of drug molecules on the nanotubes including their advantages over classical drug delivery in cancers.

KEYWORDS: Anticancer drug delivery, single walled carbon nanotubes, cancer cells

ANTICANCER THERAPEUTICS BASED ON NANOCARRIERS

According to a report from the world health organization (WHO), 7.6 million people died from cancer in the year 2005(Coughlin and Ekwueme, 2009). Despite the availability of chemotherapeutic agents, the survival rate remains low. The abnormal tumour vasculature formed by pathological angiogenesis hinders the complete delivery of the drug at the target site resulting in relapse due to incomplete eradication of malignant cells (Hillen and Griffioen, 2007). Additionally, rapidly mutating malignant cells readily develop resistance against cytotoxic agents.

RATIONALE FOR USING NANO-CARRIERS FOR ANTICANCER DRUG DELIVERY

Conventional chemotherapy which follows first order drug release kinetics where the release rate is directly proportional to the amount left in the carrier (figure**1**) has many disadvantages (Hughes, 2005).

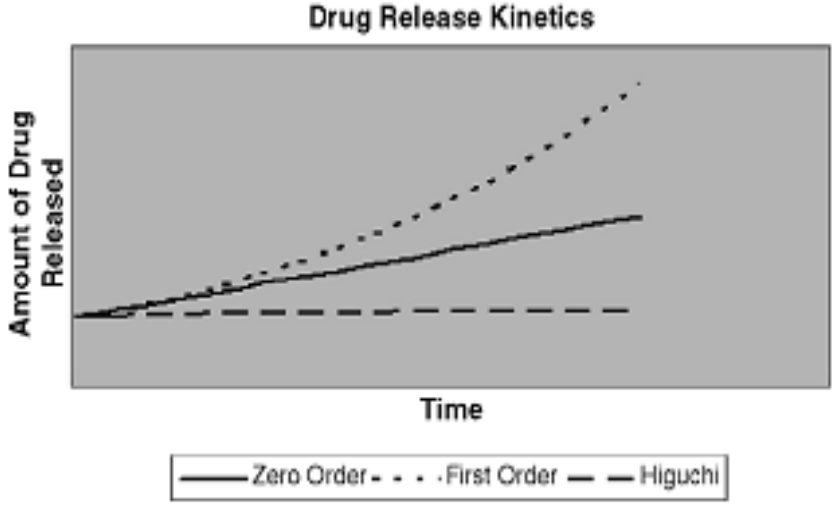

Fig. (1). Patterns of drug release profiles (Hughes, 2005).

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These include a lack of tumour tissue specificity which causes systemic distribution of drugs leading to severe systemic toxicity problems like myelosuppression. Furthermore, in conventional delivery systems, there is no control on drug release rate which is highly desirable in anticancer therapy to maintain a constant drug level at the tumour site.

To overcome these issues, nano size carriers for example carbon nanotubes, liposomes, dendrimers etc. are being used in anticancer drug delivery research. These nanocarriers follow zero order release kinetics (figure**1**) where the drug is released in a constant manner to maintain constant drug levels in the body (Cho et al., 2008). Among these nanocarriers, carbon nanotubes offer several advantages (Malam et al., 2009) over conventional delivery systems include:

- High cell penetrating ability contributing in delivering high drug payloads
- Increase circulation time of the drug in the blood
- Control the delivery of drug by releasing it at a constant rate, thus maintaining the drug levels leading to increase in efficacy of the treatments while improving the quality of life of the patient (Petrak, 2005).
- Increase in the stability of drug by preventing premature degradation or undesired conjugations such as sulfation or glucuronidation in biological milieu
- Improve the solubility and absorption of hydrophobic drugs
- Enhance the absorption of drug at the tumour site by exploiting tumour physiology. Additionally, prevent systemic toxicity of toxic drugs.

Carbon nanotubes discovered by Iijima, are cylindrical shaped rolled graphene sheets having sp^2 hybridized carbons (Iijima, 1991).

The size of nanotubes refers to the length x diameter of nanotubes which are generally of two type, armchair and zig-zag. When unrolled, the unit cells could be counted from 0, 0 to 5, 5 or 0, 0 to 9, 0 for armchair and zig- zag nanotubes respectively (figures 2 and 3) (Harris,2001).

STRUCTURE AND CHEMISTRY OF CARBON NANOTUBES

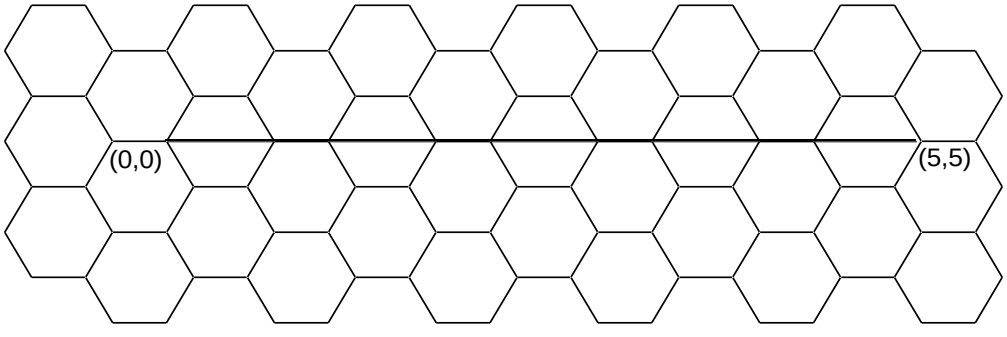

Fig. (2). Unit cells of (5, 5) armchair nanotube (8).

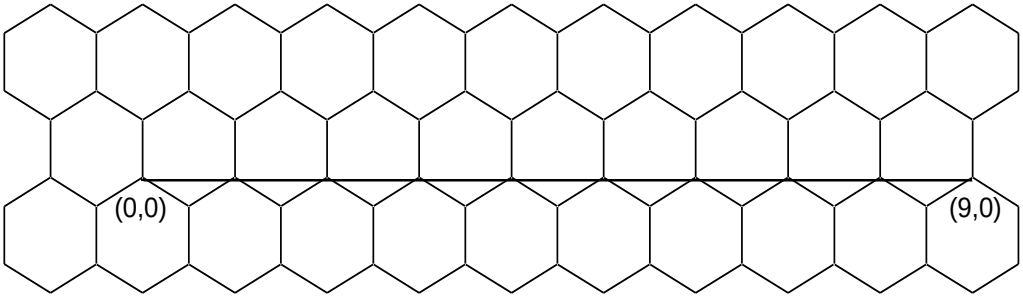

Fig. (3). Unit cells of (9, 0) zig-zag nanotube (Harris,2001).

These sheets when rolled form nanotubes which may be single walled (SWNTs) or multi walled carbon nanotubes (MWNTs). These nanotubes are further functionalized to increase the solubility in solvents as well as to prevent the formation of the bundles or aggregates.

Pristine or unfunctionalized carbon nanotubes are insoluble in either all organic or aqueous solvents. In contrast, when their ends are functionalized with functional groups such as carboxyl, amine, amide etc. they become soluble depending on the extent of functionalization and solubility in the given solvent (Bahr and Tour, 2002).

Commonly used approaches for nanotubes modification include:

(a) Covalent attachment of functional groups through chemical reactions

Covalent attachment involves generation of functional groups at the cap ends of nanotubes. For example, carboxyl group functionalization is commonly done by sonicating the mixture of nanotubes with sulphuric acid and nitric acid in appropriate amounts or treatment with sulphuric acid and hydrogen peroxide mixture (commonly named as "piranha") (Liu et al., 1998). From carboxy group, further fuctionalization can also be done. This include the treatment with thionyl chloride for generation of carbonyl group which converts into amide derivative when treated with amides. Utilizing covalent chemistry, various proteins and amino acids have been attached on either single or multi walled carbon nanotubes via diimide chemistry and 1, 3-dipolar cycloaddition reactions. In diimide reaction carboxy functionalized nanotubes are first treated with N-ethyl-N'-(3-dimethyl- amino propyl) carbodiimide hydrochloride (EDAC) to form the active intermediate O- acylisourea which when allowed to react with N-hydroxysuccinimide (NHS) resulting in an ester. The ester generated nanotubes when react with amino functionality of proteins or amino acids form an amide bond via nucleophilic substitution reaction (Jiang et al., 2004) (Gao and Kyratzis). Among anticancer agents, 10-hydroxycamptothecin has been covalently attached to multi walled nanotubes (MWNT) via intracellular enzymes susceptible ester linkage and a hydrophilic linker diaminotriethylene glycol. The system has shown a significant decrease in tumour size when tested on tumour induced mice in *in-vivo*. Additionally, the composite has shown superiority over clinical formulation of 10-hydroxycamptothecin which was taken as standard. It has also shown improved bioavailability and extended circulation time of active drug in comparison to free 10-hydroxy-camptothecin. This system could be useful in overcoming the solubility and stability problems of camptothecins in plasma (Wu et al., 2009).

(b) Noncovalent adsorption of small molecules or wrapping of polymers around nanotubes:

In this category several peptides (Ortiz-Acevedo et al., 2005), polymers (Tasis et al., 2006) and anticancer agents such as doxorubicin (Liu et al., 2007) have been tested.

(c) Filling inside the cavity of carbon nanotubes:

Under this category many small molecules such as cytochrome c3 (Tsang et al., 1995), anticancer agent cisplatin (Tripisciano et al., 2009) have been successfully tried for biological purposes.

TUMOUR TARGETING BY CARBON NANOTUBES

Drug loaded carbon nanotubes reach at the tumour sites either via passive or active targeting. First generation nanocarriers follow passive targeting methodology which is based on enhanced permeation and

retention (EPR) concept while second generation is based on active targeting.

Passive Targeting

It is based on enhanced permeation and retention (EPR) concept and exploits tumour biology in drug targeting. Tumours having size more than 2 mm^3 (~10^6 cells) are unable to take nutrients by diffusion and therefore undergo angiogenesis to continue the supply of nutrients and oxygen (Madhusudan and Harris, 2002).

In normal physiological conditions, angiogenesis involving the formation of new blood vessels from the pre-existing vasculature remains under stringent control by a balance between its activators and suppressors. But in malignancy this balance is disrupted, resulting in an "angiogenic switch" (term coined by Hanahan and Folkman, 1996) leading to over-expression of pro-angiogenic factors in the cells (Hanahan and Folkman, 1996, Makrilia et al., 2009, Kroemer and Pouyssegur, 2008).

However, the incompletely formed neovasculature always remains leaky and dilated with poor alignment of endothelial cells and absence of complete basement membrane. Moreover, due to absence of tight endothelial cell junctions (Baranwal and Alahari, 2009, Brooks et al., 2010) the pore size of blood vessels range from 100-700 nm in contrast to normal vessels where the pore size remains from 2-6 nm (Maeda, 2001, Francavilla et al., 2009). Due to pathological angiogenesis involving incomplete formation of blood vessels there always remains a hypoxic condition in the tumour tissue (Brown and Wilson, 2004). In response to hypoxia, malignant cells commence with aerobic glycolysis which contributes in decreasing the pH. This pH leads to further decrease (Helmlinger et al., 1997) with increasing distance of cells from the blood vessels. The pH drop also activates various events which contribute in seven hallmarks of cancer (Kroemer and Pouyssegur, 2008). For example, low pH due to lactic acid activates matrix metalloproteinases which degrade the basement membranes and help in invasion of malignant cells. It also suppresses the activation and maturation of cytotoxic T-lymphocytes (Dunn et al., 2006) and interferes in the functioning of natural killer cells (Smyth et al., 2002) leading to suppression in the process of tumour immunosurveillance (Fischer et al., 2007). On the other hand, normal cells undergo aerobic respiration (Swietach et al., 2007) where the pyruvic acid (generated from glucose molecules via glucose-6-phosphate) enters into the mitochondrial Kreb's cycle and produces 36 ATP molecules on per molecule of glucose. This process utilizes oxygen molecules and generates carbon dioxide which either diffuses from the cell membrane or in presence of carbonic anhydrase inside the cells and form hydrogen and bicarbonate ions. These ions are readily balanced by various transporters such as sodium-hydrogen exchangers present at the cell membranes. Additionally, due to nearness with capillaries, these ions are readily exchanged with ions in the blood to maintain the intracellular of 7.2 and extracellular pH of 7.4 (Gatenby and Gillies, 2004).

However, in case of malignancy due to absence of adequate oxygen supply and various mutations in cellular components including mitochondria (Carew and Huang, 2002) and hypoxia inducible factor genes, the pyruvic acid molecules instead of entering in Kreb's cycle start generating lactic acid molecules. These lactic acid molecules decrease the intracellular pH and when they cross the cell membranes via monocarboxylic acid transporters result in a decrease the extracellular pH. Furthermore, lactate molecules also release protons which either cross membranes via sodium-hydrogen exchange pumps or in presence of intracellular bicarbonate ions generate carbon dioxide molecules which in turn generate bicarbonate and hydrogen ions in presence of carbonic anhydrase at the membranes. These bicarbonate and H+ ions outside the cells decrease the extracellular pH. Also, due to distance from capillaries, the lack of ions exchange contributes in decreasing extracellular pH. Viewing the above discussed conditions, the carbon nanotubes based formulations could be a satisfactory attempt in releasing the drug at tumour site in a controlled fashion to kill malignant cells and thereby suppressing the tumour promoting events by these cells. Being of nano size the nanotubes could successfully deliver the drug at tumour site via enhanced permeation and retention (EPR) concept and release the drug in a controlled manner. It also increases the bioavailability of the drug. Additionally, the nanotubes acting as cargo could deliver a large amount of drug inside the malignant cells which is favourable over the delivery of few drug molecules which causes ineffective cell killing. Nanotubes acting as a drug cargo could also be fabricated with tumour targeting antibodies to further increase the retention time in the tumour interstitium. Nanomaterials, especially carbon nanotubes of size between 100-700 nm and weight more than 50 kDa can easily permeate tumour blood vessels but not in

normal ones. Additionally, due to poor lymphatic drainage, the retention time of the drug increases at the tumour site which contributes in increasing selectivity and

This tumour selective targeting is advantageous over administration of low molecular weight drugs without any carrier which leads to back diffusion of drug molecules into the blood stream leading to poor concentration at the tumour site (Iyer et al., 2006).

Furthermore, to prevent the nanocarriers from reticuloendothelial system (RES) uptake, coating of biocompatible hydrophilic polymers such as polyethylene glycol and dextran has also been performed (Monfardini and Veronese, 1998). It also extends the circulation time of drug molecules in the blood by providing stealth character. It is supposed that hydrophobic particles are more readily opsonized in comparison to hydrophilic.

Opsonin and other plasma proteins readily adsorb on the surface of hydrophobic nanocarriers and subsequently get recognized by macrophages which bind to these adsorbed opsonins via their receptors and engulf them. This process is commonly known as opsonization and engulfing by mononuclear phagocyte system (MPS) or reticuloendothelial system (RES) (Owens and Peppas, 2006).

Active Targeting

For active targeting, many nanoparticles are under clinical evaluation (Marcucci and Lefoulon, 2004) as drug retention time in the interstitium of malignant mass is controlled by the targeting ligand and not by enhanced permeation and retention property.

Drug targeting prevents non-specific distribution which causes systemic toxicity. Active targeting involves fabrication of nanocarriers by a tumour targeting moiety such as an antibody or ligand which binds to its receptors which are over or selectively expressed on tumour cells. It also increases drug efficacy while decreasing the IC50 (Peer et al., 2007). A recent example of targeted drug delivery using single walled carbon nanotubes (SWNT) is the composite of P-glycoprotein antibody (Seeger and van Veen, 2009) functionalized single walled carbon nanotubes loaded with doxorubicin (Li et al., 2010).

Here, the P-gp antibody was attached to nanotubes by covalent attachment using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Thereafter, the loading of doxorubicin was performed. The composite

decreasing toxicity. Moreover, being of large size the possibility of back diffusion is also diminished thus, allow the drug to remain in the tumour interstituim.

successfully inhibited the proliferation and maturation of K 562R human leukemia cells having over-expressed P-glycoproteins.

The decrease in cell viability was in a time dependent manner. A significant difference in the percentage of malignant cell death was observed when functionalized single walled carbon nanotubes loaded with doxorubicin system was used in comparison to free doxorubicin, P-gp antibody-SWNT, doxorubicin-SWNT and doxorubicin-human serum albumin-SWNT systems. Moreover, a controlled release fashion of doxorubicin was found when the system was incubated and irradiated with near infra-red (NIR) demonstrating that the release of drug molecules could be controlled by NIR irradiation.

This delivery system could be an excellent strategy in overcoming multidrug resistance (Gillet et al., 2007, Coley, 2009) which has become a major obstacle in cancer treatment beside of availability of P-gp inhibitors (McDevitt and Callaghan, 2007). Thus, utilization of single walled carbon nanotubes has imparted several excellent properties in the above discussed delivery system they include:

- Active targeting of tumour and avoidance of non-specific distribution
- High drug loading capacity of carbon nanotubes which deliver large amount of drug at the desired site
- Antibody linked to nanotubes leading to specific targeting of cancerous cells having over-expressed P-gp pumps and enables the system capable in overcoming drug resistance due to efflux by P-gp
- Being large, the composite cannot be expelled out by P-gp pumps in comparison to small molecules of drug alone
- Release is controlled by NIR which contributes in minimizing doxorubicin induced cardiotoxicity.

SOME NOTABLE ANTICANCER FORMULATIONS BASED ON CARBON NANOTUBES

Carbon nanotubes have shown great potential in several fields of medicine and drug delivery. Various small molecule drugs (Wu et al., 2005) DNA (Singh et al., 2005), RNA (Yeh and Hummer, 2004), peptides

(Pantarotto et al., 2004) (Pantarotto et al., 2003), proteins (Davis et al., 1998, Shi Kam et al., 2004) have been immobilized, encapsulated or covalently attached on functionalized carbon nanotubes.

Among anticancer delivery systems some notable preparations are discussed below:

(a) Biotin-Single wall carbon nanotube- paclitaxel conjugate:

This delivery system (Chen et al., 2008) (Chen et al., 2010) could be a successful replacement of cremophor which is used as a delivery vehicle to dissolve extremely hydrophobic paclitaxel. Cremophor (castor oil) has number of problems; firstly, being toxic, it causes severe hypersensitivity reactions, vasodilatation, anaphylactic shock etc. Moreover, drugs administered earlier to prevent these complications interfere in the metabolism of paclitaxel. For example, dexamethasone and the H2 blocker ranitidine are cytochrome P450 enzymes inhibitors. Secondly, cremophor causes leaching of polyvinyl chloride infusion bags used during administration.

Apart from other problems, cremophor itself imparts undesirable cytotoxic action on cells along with paclitaxel (Singla et al., 2002) . In the single walled carbon nanotube based composite, paclitaxel was covalently attached to functionalized nanotubes via a disulfide enzyme cleavable linker. For cancer selectivity, a tumour targeting ligand biotin was covalently attached to the nanotubes. The system has shown a significant decrease in the viability of L1210FR leukemia cell lines (over-expressing biotin receptors) at less IC50 in comparison to free taxol. The conjugate was internalized inside the malignant cells via receptor mediated endocytosis and the linker released the free paclitxel molecules in a controlled manner after the hydrolytic cleavage. The free drug molecules inhibited tubulin polymerization blocked the mitosis and subsequently triggered the apoptotic pathway resulting in cell death (McGrogan et al., 2008).

(b) Single wall carbon nanotube-Platinum (IV)-Folic acid conjugate (SWNT-Pt (IV)-Fl conjugate):

The amino functionalized SWNT-Pt (IV)-Fl conjugate has shown a significant decrease in malignant cells viability over cisplatin alone. It was designed to target folic acid receptors of the malignant cells. Additionally, the Platinum (IV) complex (c,c,t- [Pt(NH3)2Cl2(O2CCH2CH2-O2H)(O2CCH2CH2CONH-Polyethylene glycol –Folic acid) containing polyethylene glycol increased the metabolic stability and aqueous solubility of the platinum prodrug. Simultaneously, the folate group provided specific tumour targeting which is desirable to decrease the systemic toxicity of platinum based anticancer agents. The conjugate remains stable in plasma and releases the active moiety cisplatin which binds to DNA (Reedijk, 1987) only inside the folate receptors expressing malignant cells after binding of folic acid to folate receptors and endocytosis (Dhar et al., 2008).

(c) Pegylated single wall carbon nanotubes- doxorubicin system:

This system has shown a pH dependent loading of doxorubicin on polyethylene glycol coated water soluble single walled carbon nanotubes.

Due to increase in protonation of amino groups of the molecule at low pH, the hydrophilicity and solubility increased and thus reduced the hydrophobicity resulting in decreased π-stacking interactions between doxorubicin and single walled carbon nanotubes. Additionally, the system showed less decrease in cell viability over free drug when tested on U87MG cell lines. Thus, the system is less toxic in comparison to free drug (Liu et al., 2007).

(d) Platinum IV complex with amine functionalized SWNT:

The delivery system has proved the utility of single walled carbon nanotubes again. Covalent attachment of Pt (IV) complex c,c,t-[Pt(NH3)2Cl2(OEt)(O2CCH2CH2CO2H)] with amine functionalized nanotube significantly decreased the viability of NTera-2 testicular cancer cells. Nanotubes after entering inside the cells via endocytosis released the active molecule which is otherwise ineffective on these cell lines (Feazell et al., 2007).

(e) Carbon nanotubes are also reported in the targeted treatment of bladder cancer. In this approach, tumour specific single walled carbon nanotubes-annexin V and phosphatidylserine conjugate was delivered intravescially followed by a controlled near infra-red radiation. After 24h hours of treatment, no tumour remains were visible on the bladder cell wall. Also, there was no damage to the healthy bladder cell wall. Moreover, no toxicity was detected throughout the treatment (Virani et al., 2018).

(f) Multi walled carbon nanotubes are also reported for the loading of anticancer drug

Busalfan. The study demonstrated the loading efficiency of multi walled carbon nanotubes with improved bioavailability and reduced systemic toxicity at the cancer site (Ghoshal et al., 2016).

CONCLUSIONS AND FUTURE PERSPECTIVES

Carbon nanotubes have immense potential to be used in novel drug delivery systems and are currently under investigation. The advantage of drug loading capacity and organ targeting ability makes them remarkable nanocarriers. At the same time, the controlled drug releasing ability makes them beneficial in anticancer drug delivery in order to maintain a constant plasma drug levels while preventing side effects. Moreover, the delivery system could help in improving the bioavailability as well as the stability of the drug in biological milieu. Concluding, the impact of carbon nanotubes in anticancer formulation designing will greatly increase if proper functionalization and drug attachment methods along with their biological studies can be done worldwide.

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