INVITED REVIEW ARTICLE

Octa-ammonium POSS-conjugated single-walled carbon nanotubes as vehicles for targeted delivery of paclitaxel

Naghmeh Naderi[1,2], Seyed Y. Madani[1], Afshin Mosahebi[3] and Alexander M. Seifalian[1,3]*

[1]UCL Centre for Nanotechnology & Regenerative Medicine, University College London, London, UK; [2]Institute of Life Science, College of Medicine, Swansea University, Swansea, UK; [3]Royal Free London NHS Foundation Trust, London, UK

Abstract

Background: Carbon nanotubes (CNTs) have unique physical and chemical properties. Furthermore, novel properties can be developed by attachment or encapsulation of functional groups. These unique properties facilitate the use of CNTs in drug delivery. We developed a new nanomedicine consisting of a nanocarrier, cell-targeting molecule, and chemotherapeutic drug and assessed its efficacy *in vitro*.

Methods: The efficacy of a single-walled carbon nanotubes (SWCNTs)-based nanoconjugate system is assessed in the targeted delivery of paclitaxel (PTX) to cancer cells. SWCNTs were oxidized and reacted with octa-ammonium polyhedral oligomeric silsesquioxanes (octa-ammonium POSS) to render them biocompatible and water dispersable. The functionalized SWCNTs were loaded with PTX, a chemotherapeutic agent toxic to cancer cells, and Tn218 antibodies for cancer cell targeting. The nanohybrid composites were characterized with transmission electron microscopy (TEM), Fourier transform infrared (FTIR), and ultraviolet–visible–near-infrared (UV–Vis–NIR). Additionally, their cytotoxic effects on Colon cancer cell (HT-29) and Breast cancer cell (MCF-7) lines were assessed *in vitro*.

Results: TEM, FTIR, and UV–Vis–NIR studies confirmed side-wall functionalization of SWCNT with COOH-groups, PTX, POSS, and antibodies. Increased cell death was observed with PTX–POSS–SWCNT, PTX–POSS–Ab–SWCNT, and free PTX compared to functionalized-SWCNT (f-SWCNT), POSS–SWCNT, and cell-only controls at 48 and 72 h time intervals in both cell lines. At all time intervals, there was no significant cell death in the POSS–SWCNT samples compared to cell-only controls.

Conclusion: The PTX-based nanocomposites were shown to be as cytotoxic as free PTX. This important finding indicates successful release of PTX from the nanocomposites and further reiterates the potential of SWCNTs to deliver drugs directly to targeted cells and tissues.

Keywords: *carbon nanotube*; *drug delivery*; *nanotechnology*

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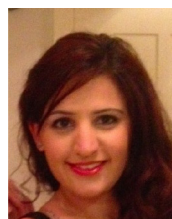
Dr Naghmeh Naderi is an academic clinical lecturer at Swansea University College of Medicine, and a specialist registrar in plastic surgery at the Welsh Centre for Burns and Plastic Surgery. She obtained her medical degree from University Utrecht in 2007 and went on to complete her foundation and core surgical training in the United Kingdom. She became a member of the Royal College of Surgeons of Edinburgh in 2009 and went on to obtain an MSc degree in Nanotechnology & Regenerative Medicine from University College London (UCL) in 2011. She is currently pursuing her PhD at Swansea University. She has specialized in tissue engineering, regenerative medicine, and nanotechnology.

Dr Seyed Y. Madani qualified as a pharmacist from the University of Bradford. He then worked as a pharmacist for several years which made him interested in the field of oncology and the action of chemotherapeutic drugs for the treatment of cancer. Subsequently, he completed his PhD from UCL, researching on the application of nanoparticles for the treatment of cancer. He is currently pursuing his MBA at Edinburgh Business School so as to direct, manage, and lead a team of scientists researching the diagnostics and treatment of cancer.

Dr Afshin Mosahebi is a consultant plastic surgeon at the Royal Free Hospital and senior lecturer in plastic surgery at UCL. He qualified from Kings College Medical School in London and obtained a PhD in nerve regeneration from UCL and an MBA from Warwick

According to World Health Organization (WHO) reports, there were an estimated 12.4 million new cases of cancer worldwide in 2008 and 7.6 million deaths as a result (1). It has also been statistically calculated that more than one in three people will develop some form of cancer in their life time (2). Despite improvements in medical care over the last century, mortality of cancer exceeds 155,000 annually (3). In spite of the improvements in the efficiency of treatments over the last few decades, the majority of conventional chemotherapeutic formulations pose multiple problems such as systemic toxicity and a destructive 'by-stander' effect to neighbouring cells, causing nephrotoxicity, neurotoxicity, vascular toxicity, infertility, myocardial infarction, and thrombo-embolic complications as well as hair loss, nausea, and vomiting (4).

Due to advances in synthetic chemistry over the last few years, different biological nanomaterials have been developed, which can be used for a variety of biological therapies such as drug delivery, cancer diagnosis, treatment, and imaging. This group of nanomaterials include quantum dots (QD), dendrimers, CNTs, gold and silver nanoparticles, liposomes, and micelles. Nanomaterials exhibit advanced physical properties, which show promise for various biological applications (5–8). They have shown great potential in cancer therapeutics and diagnostics, leading to improved tissue distribution and prolonged blood circulation times of cancer drugs (9). Among nanomaterials, CNTs are probably one of the safer materials due to their lack of heavy metal composition (10). They are insoluble in water but can be solubilized by chemical modification. Effective purification of the CNTs is vital to avoid structural damage and undue cytotoxicity. Typically, CNTs are classified as either single-walled carbon nanotubes (SWCNTs) – consisting of one layer of cylinder graphene or multi-walled carbon nanotubes (MWCNTs) – containing several concentric graphene sheets (11). CNTs have unique physical and chemical properties such as high aspect ratio, ultra-light weight, large surface area, high mechanical strength, high electrical conductivity, and high thermal conductivity (11). Among other areas, CNTs are currently used in cancer photothermal therapy, photodynamic therapy, drug delivery, and gene therapy. Novel properties can be developed to match the clinical need by attachment or encapsulation of functional groups to the CNT. These unique properties facilitate the use of CNTs as a drug delivery system (DDS), designed to improve the distribution and performance of drug molecules.

The design of new strategies for the delivery of drugs into cells is necessitated by the poor cellular penetration of many biological cargoes. CNTs have emerged as promising nanocarriers in living systems. It has been shown that CNTs can serve as highly efficient vehicles to transport a wide range of molecules across membranes into living cells with little cytotoxicity (12). CNTs, functionalized with

Business School. He was the first plastic surgery fellow from the United Kingdom at the world famous Memorial Sloan Kettering Cancer Centre, Manhattan, New York City. Ash is the deputy editor of *British Journal of Plastic Surgery* (JPRAS). He is involved in pioneering research in the use of stem cell for tissue regeneration and nanoparticle for drug delivery.
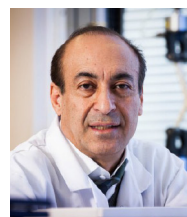
Alexander M. Seifalian is professor of Nanotechnology and Regenerative Medicine. His current projects have led to the development of cardiovascular implants using nanomaterials and stem cell technology, and the development of organs using tissue engineering and nanoparticles for the detection and treatment of cancer. He was awarded the top prize in the field of nanomaterials for cardiovascular implants in 2007, and in 2009 received a Business Innovation Award from UK Trade & Investment. He received the most innovative new product award in 2012 for the 'synthetic trachea' from European Life Science, Germany.

antibodies or ligands recognizing specific antigens or receptors, and simultaneously carrying anti-tumour drugs, can be directed to the surface of cancer cells. With very high surface area per unit weight, CNTs provide higher capacity of drug loading compared to conventional liposome and dendrimer drug carriers (13). The underlying process of functionalization involves the selective breaking of C=C bonds in the CNT (typically by oxidation), resulting in carboxyl groups that can then be used as subsidiary sites for addition reactions. By virtue of spontaneous internalization (14–16), that is, endocytosis, the CNTs are taken up by the cells before the anti-tumour drugs are cleaved off and targeted delivery is realized. This concept may avoid systemic toxicity and destructive effects to healthy neighbouring cells when cytotoxic drugs are used.

Recently, investigators have developed novel CNT-based tumour-targeting DDSs (Table 1). These DDSs consist of functionalized CNTs and anti-cancer drugs with or without tumour-targeting ligands/antibodies. Although initially investigated on cultured cells *in vitro*, an increasing number of *in vivo* studies investigating the role of CNT conjugates have been published. Data from these studies suggest that toxic side effects of chemotherapeutics can be significantly reduced by incorporation into CNT conjugates.

In this study, we have developed a new nanomedicine comprising a SWCNT nanocarrier, polyhedral oligomeric silsesquioxanes (POSS) molecules to increase solubility and biocompatibility, a cell-targeting antibody, and a chemotherapeutic drug. Specifically, the efficacy of this SWCNT-based nanoconjugate system is assessed in

Table 1. CNT-based tumour-targeting drug delivery systems *in vitro* unless indicated with * *in vivo*

CNT	Drug	Tumour-targeted modules	Tumour	Cellular response	Ref.
SWCNT	Streptavidin	Biotin	Promyelocytic leukaemia (HL60) and Human T-cells	Extensive cell death	(17)
SWCNT	DOTA or DFO	E4G10	LS174T colon cancer xenograft in nude mice	↓ Tumour vasculature	(18)
SWCNT	DOX	Cyclic RGD and PL-PEG	MCF-7 breast cancer and U87MG glioblastoma cells	↑ Cytotoxicity	(13)
MWCNT	TCV	H22P	Murine H22 hepatoma	↑ Tumour cure rate	(19)
SWCNT	Platinum (IV) → cisplatin intracellularly	Folic acid	Nasopharyngeal epidermoid cancer (KB), testicular cancer (NTera-2), and choriocarcinoma (JAR)	↑ Cytotoxicity	(20)
SWCNT	Taxoid	Biotin	L1210FR and L1210 leukaemia cells	↑ Cytotoxicity	(21)
SWCNT	Paclitaxel	PEG	Murine 4T1 breast cancer cells	↓ In tumour size	(22)
MWCNT	Carboplatin	–	EJ28 bladder cancer cells	↑ Cytotoxicity	(23)
MWCNT	DOX	Pluronic F127	MCF-7 breast cancer cells	↑ Cytotoxicity	(24)
*SWCNT	Cisplatin	EGF	Murine HNSCC tumours	↓ In tumour size	(25)
SWCNT	DOX	Folic acid	HeLa cervical cancer cells	↑ Cytotoxicity	(26)
*MWCNT	HCPT	–	MKN-28 gastric cancer cells, Murine H22 hepatoma	↑ Anti-tumour effect	(27)
SWCNT	DOX	Monoclonal antibody	WiDr colon cancer cells	Intracellular SWCNT-DOX uptake and release	(28)
SWCNT	Integrin $\alpha_v \beta_3$ MA	PL-PEG	U87MG glioblastoma cells	↑ Cytotoxicity	(29)
SWCNT	DOX	PL-PEG	SCID mice bearing Raji lymphoma	↑ Cytotoxicity compared to free DOX	(30)
MWCNT	Ricin A chain protein	Anti-HER2 antibody	HER2/neu-R+ breast cancer cells	↑ Cytotoxicity	(31)
SWCNT	DOX	PEG	Murine B16 F10 melanoma cells	Similar to, but more selective cytotoxicity than free DOX	(32)
SWCNT MWCNT	Paclitaxel	PEG	HeLa cervical and MCF-7 breast cancer cells	↑ Cytotoxicity	(33)
SWCNT	DOX	P-glycoprotein antibody	K562 multiresistant leukaemia cells	↑ Cytotoxicity	(34)

DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DFO, desferrioxamine B; PL-PEG, phospholipid bearing polyethylene glycol; RGD, arginine–glycine–aspartic acid; HNSCC, head and neck squamous cell carcinoma; DOX, doxorubicin; HCPT, 10-hydroxy-camptothecin; TCV, tumour cell vaccines; EGF, epidermal growth factor; H22p, tumour lysate protein; MA, monoclonal antibody; ↑, increase.

the targeted delivery of paclitaxel (PTX) to HT-29 and MCF-7 cancer cell lines. POSS is derived from a class of compounds closely related to silicones and has the chemical composition of a hybrid, intermediate between that of silica and silicone. Each POSS molecule contains bonded reactive functionalities suitable for polymerization or grafting. The chemical diversity of POSS technology is very broad and, in contrast to pristine CNTs, POSS molecules are cytocompatible and hence suitable for the synthesis of nanocomposites for biomedical applications (35, 36). Experiments have not shown any significant difference in cell viability, adhesion, and proliferation between POSS nanocomposites and standard cell culture plates. As the POSS molecules have better reactivity and solubility, functionalization of POSS molecule with CNTs can substantially enhance the solubility and processability of this nanocarrier. PTX (Fig. 1) is a mitotic inhibitor and a potent cytotoxic drug with anti-tumour activity against a variety of *in vitro* and *in vivo* tumours (37), including colon cancer (38). PTX is also a very effective drug in treating breast cancer (39) having demonstrated some survival

advantage over previous standard regimens both in the adjuvant and advanced setting of breast cancer disease (40). PTX binds to the N-terminus of β-tubulin and stabilizes microtubules arresting the cell cycle at the G_2/M phase. However, it has undesirable side effects such as myelosuppression, neuropathy, myalgias, fatigue, alopecia, diarrhoea, mucosal toxicity, and skin and nail changes (41). Furthermore, the poor aqueous solubility of PTX is
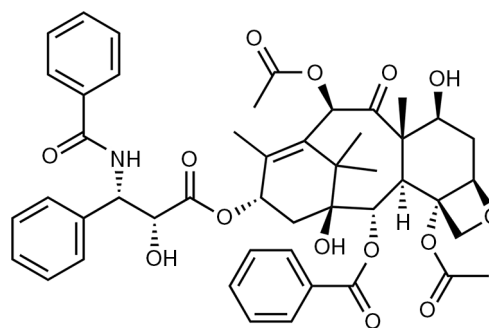

Fig. 1. Molecular structure of paclitaxel.

a major limitation of its success clinically. The efficient delivery, preservation of drug–molecular bioactivity, and desirable loading and release kinetics of drug molecules towards targets are the driving forces in the design of DDSs. CNT-based drug delivery may offer a solution to these limitations. Targeted drug delivery is the effective strategy to provide therapeutic concentrations of drugs at the targeted cells rather than the non-targeted cells by a variety of functionalized drug carriers, which thus improve the therapeutic efficacy for targeted tissues and reduce the side effects. The efficient delivery, preservation of drug-molecular activity, and desirable loading and release kinetics of drug molecules towards targets are the driving forces in the design of a targeted DDS. To overcome the limitations of passive targeting, we programmed our carbon nanocarriers so that they actively bind to the MCF-7 and HT-29 cancer cells. This binding was achieved by attaching a targeting antibody – Tn antigen monoclonal antibody – to the surface of the carbon nanocarrier. Targeted nanocarriers will recognize and bind to target cells through ligand–receptor interactions, and are then internalized before the drug is released inside the cell.

The blood group precursors T (Thomsen–Friedenreich) and Tn epitopes are shielded in healthy and benign-diseased tissues but uncovered in approximately 90% of carcinomas. T and Tn glycoproteins are specific, auto-immunogenic pancarcinoma antigens. Tn epitopes are cell and tissue adhesion molecules, essential in invasion by and metastasis of carcinoma. Approximately, 88% of breast and 85% of colon carcinomas express Tn epitopes on their cell surface (42). In the HT-29 cell line, approximately 70% of the cells express the Tn antigen (43). Among different breast cancer cell lines, MCF-7 cells present the highest level of Tn antigens (approximately 170 antigen units per mg protein) (44).

Materials and methods

Experimental agents
Octa-ammonium POSS was purchased from Hybrid Plastics (Hattiesburg, MS, USA). SWCNT, PTX, 3-dimethylaminopropyl-N-ethylcarbodiimide hydrochloride (EDC, 99%), H_2SO_4 (95%), HNO_3 (70%), N-hydroxy-succinimide (NHS) were purchased from Sigma–Aldrich (Dorset, UK). Dulbecco's modified Eagle medium (DMEM), foetal calf serum, phosphate-buffered sulphate (PBS), bovine serum albumin, and trypsin 0.25% were purchased from Invitrogen (Paisley, UK). Tn 218 antibody, Human colorectal cancer cell (HT-29) and Human Breast cancer cell (MCF-7) lines were purchased from Abcam (Cambridge, UK).

Improving solubility of SWCNTs by functionalization
According to the manufacturers, SWCNTs were obtained by chemical vapour deposition with >75% purity; their diameter ranged between 0.7 and 1.3 nm and length between 450 and 2,300 nm. A quantity of 5 mg of SWCNT was mixed with 10 ml of concentrated nitric acid (HNO_3) and 20 ml of concentrated sulphuric acid (H_2SO_4). The mixture was subsequently refluxed at 120°C for 120 min. The f-SWCNTs were washed through a 0.22-μm filter (Millipore Ltd, Watford, Hertfordshire WD18 8YH, UK, Sigma–Aldrich) with distilled water four times until the pH was normal. This process allowed for side-wall covalent functionalization with carboxylic acid groups (−COOH), rendering them water dispersible. Additionally, this would allow for further modification. Subsequently, the samples were sonicated (2 h) to ensure a homogeneous suspension of the SWCNTs in distilled water.

Conjugation of octa-ammonium POSS to SWCNTs
In this study, we reacted octa-ammonium POSS with the f-SWCNTs to give a soluble hybrid material for targeted drug delivery: SWCNT–POSS nanocomposite. The cross-linker EDC was used to covalently conjugate octa-ammonium POSS molecules onto carboxylated CNTs. EDC is a zero-length cross-linker widely used in molecular conjugations. The conjugation reactions occur in two sequential steps. EDC first reacts with a carboxyl group, forming an amine-reactive O-acylisourea intermediate, which subsequently reacts with an amine group to produce a stable amide bond. However, the O-acylisourea intermediate is unstable and susceptible to hydrolysis, resulting in low coupling efficiency. The addition of NHS stabilizes the reaction by converting the intermediate to a semi-stable amine-reactive NHS ester and increasing the coupling efficiency by 10- to 20-fold.

Conjugation of paclitaxel to POSS–SWCNT nanocomposites
PTX was modified by succinic acid (Aldrich), adding a carboxyl acid group on the molecule at the C′-2OH position. Succinic acid is a bis(carboxylic acid) moiety and is reacted on an equimolar basis with the hydroxyl group in PTX to form a PTX–succinic acid conjugate, leaving one free carboxyl group for further conjugation. Briefly, PTX (1 mg, 0.00118 mM) and succinic acid (0.14 mg, 0.00118 mM) were dissolved in 0.3 ml of anhydrous DMSO and 1 ml of anhydrous DCM. To the resulting mixture, EDC (0.2 mg) and 4-Dimethylaminopyridine (DMAP) (0.1 mg) were added. The reaction was carried out with continuous stirring for 24 h at 5°C. The resulting solution was filtered to remove dicyclohexylurea obtained as a by-product during the reaction. PTX–succinic acid conjugate was precipitated using diethyl ether and dried under vacuum. Subsequently, 0.5 mg of EDC and 0.3 mg NHS were added to this solution. The mixture was stirred at 5°C for 12 h, followed by the addition of 1 mg of octa-ammonium POSS–SWCNT nanocomposite. The mixture was stirred at room temperature for an additional 2 h, followed by

centrifugation and filtration through a 10-kDa filter (Amicon ultra-4 centrifugal filter unit), at 5°C for 50 min to remove free PTX and excess EDC and NHS.

Conjugation of antibodies to PTX–POSS–SWCNT nanocomposites

A volume of 1 ml of the PTX–POSS–SWCNT complexes in water with a concentration of 1 mg/ml were mixed with 5 ml sodium phosphate buffer (50 mM, pH 8.5). Next, 0.5 mg of EDC and 0.3 mg of NHS were added and the mixture was incubated for 5 min at room temperature. Subsequently, 0.05 mg of mouse Tn antigen monoclonal antibody was added to the solution, and the reaction allowed to proceed for 2 h at room temperature while stirring. Finally, it was centrifuged and filtrated using 100 kDa filters (Amicon ultra-4 centrifugal filter unit) at 5°C for 50 min to remove excess EDC and NHS and free PTX–POSS–SWCNT complexes. Distilled water was added to the precipitate and it was filtered and centrifuged again.

Transmission electronic microscopy

The qualitative post-purification assessment of the SWCNTs was carried out using Philips CM30 TEM operating at 300 keV at low beam intensity (beam width of 4–5 cm) and short exposure time (1–2 min).

Contact angle measurements and surface hydrophobicity

To evaluate the effect of the functionalization on the hydrophobicity of the SWCNTs, surface properties were studied pre- and post-functionalization using a drop-shape analysis system (DSA100, Hamburg, Germany). This enabled the steric contact angle measurement to be determined using the sessile drop method. In this experiment, the same quantity of functionalized and non-functionalized SWCNT powder was placed on the surface of solid graphite, which has the same chemical component as SWCNT. A sessile drop of water (10 μL) was deposited on the surface of functionalized and non-functionalized surfaces and the angle of water droplets on the surface was measured. The measurements were repeated at three different areas on each of the surfaces.

FTIR spectroscopy

For the Fourier transform infrared (FTIR) spectral measurements, a JASCO FTIR system was employed. A volume of 10 μl of these samples at a concentration of 1 mg/ml was placed on the FTIR mounting crystal. The samples were left to dry on the crystal to prevent interference of water molecules with the obtained spectra. Background spectra were obtained for each measurement before placing the samples on the FTIR crystal. Each spectrum was repeated once.

UV–Vis–NIR

UV–Vis–NIR (ultraviolet–visible–near-infrared) spectrophotometer is used for optical absorbance and reflectance measurements in the wavelength range 175–3,300 nm. UV–Vis–NIR spectroscopy can be used for qualitative and quantitative analyses of materials. In this study, UV–vis–NIR was used to characterize nanocomposites after conjugation of PTX and antibodies and compared −COOH functionalized SWCNTs with pristine SWCNTs. The spectra in the UV–Vis–NIR range were taken using a JASCO V-570 spectrometer, and UV–Vis spectra were recorded on a VARIAN Cary 300 spectrophotometer using a quartz cell with a path length of 10 mm. Each measurement was repeated three times.

In vitro assessment of SWCNT nanocomposites in cancer

The efficacy of the nanocomposites was assessed in two cancer lines: HT-29 human colon adenocarcinoma grade II cancer and MCF-7 human breast adenocarcinoma cell lines. We used DMEM containing 2 mM L-glutamine, which was modified to contain 10% foetal bovine serum, and 1% penicillin/streptomycin. For the cell studies, HT-29 and MCF-7 cells were grown on tissue culture flasks in routine aseptic cell culture conditions (37°C, 5% CO_2/air, humidified atmosphere) until 70–80% confluent. At this point, cells were enzymatically disaggregated (Trypsin 1 mg/ml) and used to set up six 96-well plates in total, with each well containing 10,000 cells.

Cell toxicity assay

Three 96-well plates were used with each well containing 10,000 cells. Each plate was divided by wells containing either HT-29 cells or MCF-7 cells. The experimental groups tested for each plate were: Ab–PTX–POSS–SWCNTs, PTX–POSS–SWCNTs, and POSS–SWCNTs conjugates and the free drug/vehicle controls PTX and COOH f-SWCNTs. For each sample, three different concentrations were used: 2, 4, and 6 μg/ml. Cells alone served as baseline controls. The metabolic activity of cultured HT-29 and MCF-7 cell lines was quantified using AlamarBlue® viability assay. Briefly, control media were removed; the cells were rinsed with PBS and 200 μl of a 10% AlamarBlue® medium prepared in fresh media was added to each well. Following 3 h incubation, AlamarBlue® fluorescence was quantified at the respective excitation and emission wavelength of 540 and 595 nm. Wells containing medium and AlamarBlue® without cells were used as blanks. The mean fluorescent units for the six replicate cultures were calculated for each exposure treatment, and the mean blank value was subtracted from these. Time points in this experiment were 24, 48, and 72 h after addition of nanocomposites and controls to the well plates containing the two cell lines.
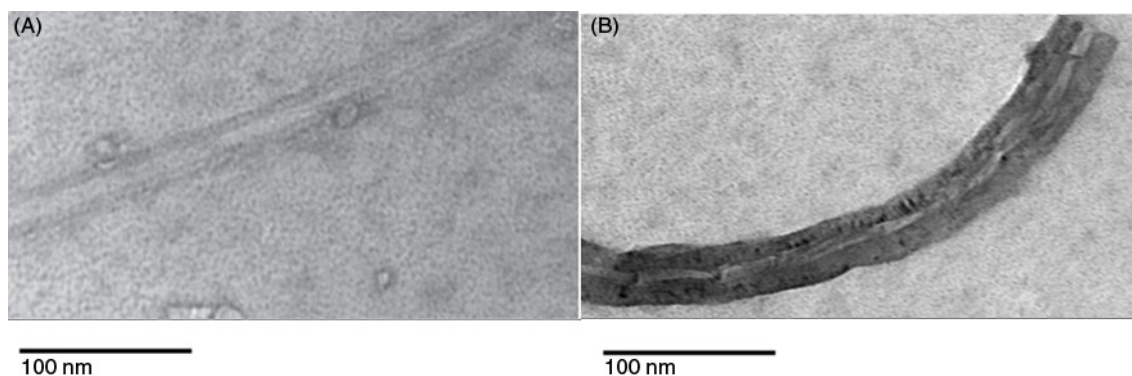
Fig. 2. TEM images of pristine (A) and −COOH functionalized SWCNTs (B).

Statistical analysis

Statistical analysis was performed using Prism software. Two-way ANOVA with Bonferroni post-test was used to show statistical significance between different concentrations, time points, F-SWNT, cell-only controls, and conjugates.

Results

Transmission electronic microscopy

By comparing the TEM images of the pristine SWCNTs and the SWCNTs treated with 1:3 ratio of HNO_3:H_2SO_4, it can be seen that the acid-treated SWCNT has obtained a thick black layer on its surface. The presence of a thick black layer could be due to the −COOH group (Fig. 2); however, this was confirmed by the graph obtained from FTIR.

Contact angle measurements

Pre-functionalization, the contact angle was $86.1° \pm 0.60°$, thereby indicating the hydrophobic nature of the material pre-functionalization. Post-functionalization, the contact angle measurements dramatically reduced to $18.5° \pm 0.63°$ (Table 2).

FTIR spectroscopy

The FTIR spectra, used to identify the hydroxyl group-terminated moieties, POSS molecules, and antibodies covalently bonded to the sidewalls of the SWCNTs, are shown in Fig. 3. The peaks are in the 1,070-cm^{-1} region,

which we attributed to the C–O bond stretches of the nanotube–O–C and C–OH units.
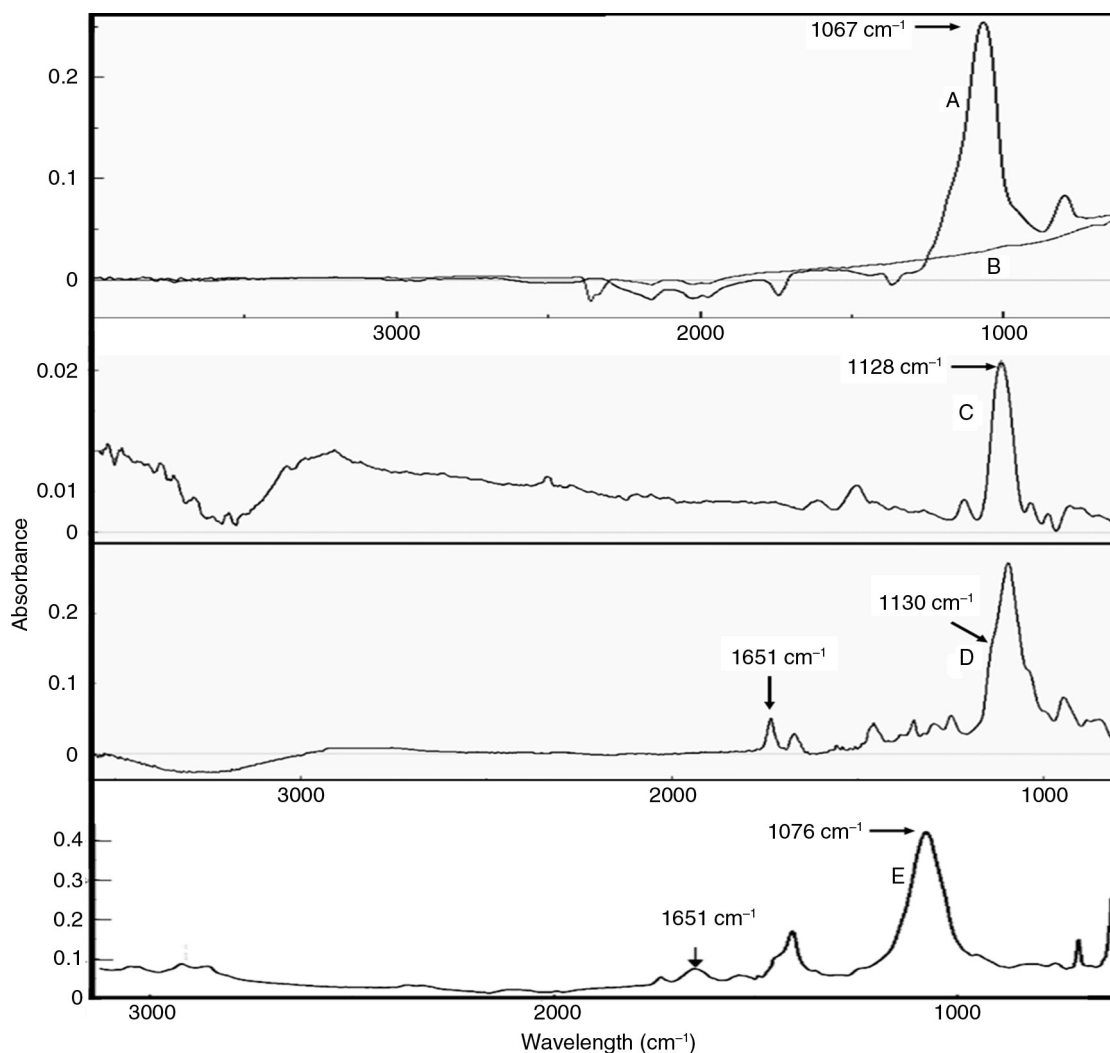
UV–Vis spectroscopy

The peak at 270 nm is suggestive of sidewall functionalization of SWCNTs with PTX and the antibody (Fig. 4).

Cell toxicity assay in vitro

To study the effect of PTX–POSS–SWCNTs nanocomposites complexes by MCF-7 and HT-29 cell lines and investigate the suitability of functionalized SWCNTs as a DDS, HT-29 and MCF-7 cells were incubated with the nanotube–drug complexes for 24, 48, and 72 h. AlamarBlue® cell viability assay was used to determine the metabolic activity of these cells. Three different concentrations were used for each nanocomposite and free PTX, and −COOH functionalized SWCNTs. There was no statistical difference in cell viability between the different concentrations of the nanocomposites and controls at any time point. In both cell lines, increased cell death was observed with free PTX and PTX containing nanocomposites compared to nanocomposites not containing PTX, cell-only controls, and −COOH functionalized SWCNT at 48 and 72 h time points. At all time intervals, there was no significant cell death in the POSS–SWCNT samples compared to cell-only controls. In the MCF-7 cell line, f-SWCNTs were associated with a significant increase in cell death compared to cell-only controls at the 72 h time point ($p = 0.001$). This difference was not statistically significant in the HT-29 cell line ($p = 0.07$; Figs. 5 and 6).

Discussion

CNTs and their derivatives are likely to make ideal candidates for application in biomedical science due to their unique physical properties, chemically modifiable surfaces, large surface areas, and tuneable length. The application of CNTs in the treatment and diagnosis of cancer is promising. It is conceivable that all real-world application of CNTs will involve some level of chemical functionalization. CNT bioconjugates can be adequately functionalized

Table 2. Physical properties of pre- and post-functionalized SWCNTs

| Physical properties of SWCNTs	Pre-functionalization (mean $\pm$ SD)	Post-functionalization (mean $\pm$ SD)	P
Contact angle	86.1 ± 0.60	18.5 ± 0.63	< 0.05

SD, standard deviation; SWCNT, single-walled carbon nanotube.

Fig. 3. FTIR spectra of: (A) −COOH functionalized SWCNTs versus (B) pristine SWCNTs. The strong peak at 1,067 cm^{-1} corresponds to C=O groups on the −COOH functionalized SWCNTs. (C) Octa-ammonium POSS and (D) octa-ammonium POSS–SWCNT conjugates. The strong peak at 1,128 cm^{-1} in C corresponds to the SiO groups on the POSS molecule. A smaller peak is seen at 1,130 cm^{-1} in D, corresponding to SiO groups on the POSS molecule attached to SWCNT. The peak at 1,650 cm^{-1} in D correlates to the amide bond between the POSS molecule and SWCNTs. (E) PTX–Ab–SWCNT conjugates. The peak at 1,076 cm^{-1} corresponds to C=O groups on f-SWCNTs, whereas the peak at 1,651 cm^{-1} corresponds to the amide bonds between SWCNTs and the antibodies.

to target specific cancer cells for both diagnostic and therapeutic purposes, leading to significant reduction of toxic side effects and better therapeutic targeting of chemotherapeutic drugs. General features of tumours include leaky blood vessels and poor lymphatic drainage. In contrast to free drugs that diffuse non-specifically, a nanocarrier can extravasate into the tumour tissues via the leaky vessels. Furthermore, the dysfunctional lymphatic drainage retains the accumulated nanocarriers and allows them to release drugs into the vicinity of the tumour cells.

We have shown that PTX–SWCNTs affords similar treatment efficacy to free PTX *in vitro*, evidenced by the significant cell death caused by PTX-containing nanocomposites.

The poor water solubility of various cancer therapeutic drugs limits their clinical applications. Cremophor EL is a commonly used reagent to disperse PTX and other drugs in saline for administration. However, its toxic effects have been noted in both animal models and patients (45). Previous reports (46) have shown short blood circulation times for free PTX with significant decline in PTX levels 11 min post-injection at 5 mg/kg-injected dose. PTX in Taxol® is known to be cleared from the blood and taken up by various organs, especially kidney and liver for rapid renal and faecal excretion with very low tumour uptake (46). Branched PEGylation of PTX via similar ester linkage as in SWNT–PTX conjugates affords water solubility of PTX. However, the blood circulation time is still short.
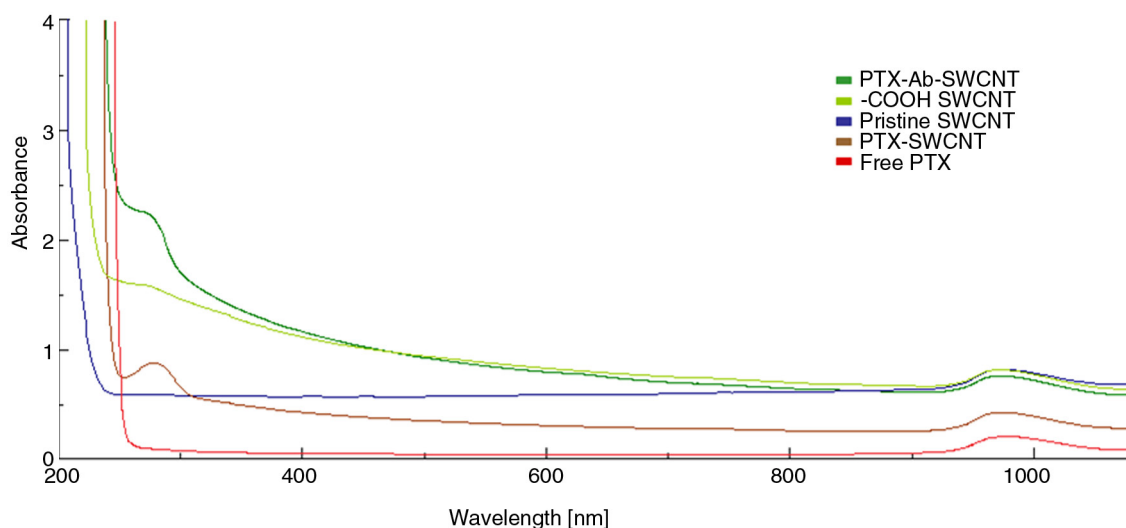
Fig. 4. UV–Vis–NIR spectra for PTX–Ab–SWCVNT and PTX–SWCNT conjugates, −COOH functionalized SWCNTs, pristine SWCNTs, and free PTX. The peak at 270 nm is suggestive of side-wall functionalization of SWCNT with PTX and antibody.

Polyethylene glycol (PEG)–PTX remains a relatively small molecule that tends to be rapidly excreted via the kidney and renal route. This leads to little advantage of PEGylation of PTX over Taxol® in tumour uptake and treatment efficacy (47).

Xie et al. used self-assembled biodegradable nanoparticles for the delivery of PTX. They investigated the *in vitro* release profile by high-performance liquid chromatography and showed sustained release of PTX for more than 20 days. *In vitro* cellular particle uptake and cytotoxicity to C6 glioma cell line seemed to be higher than that
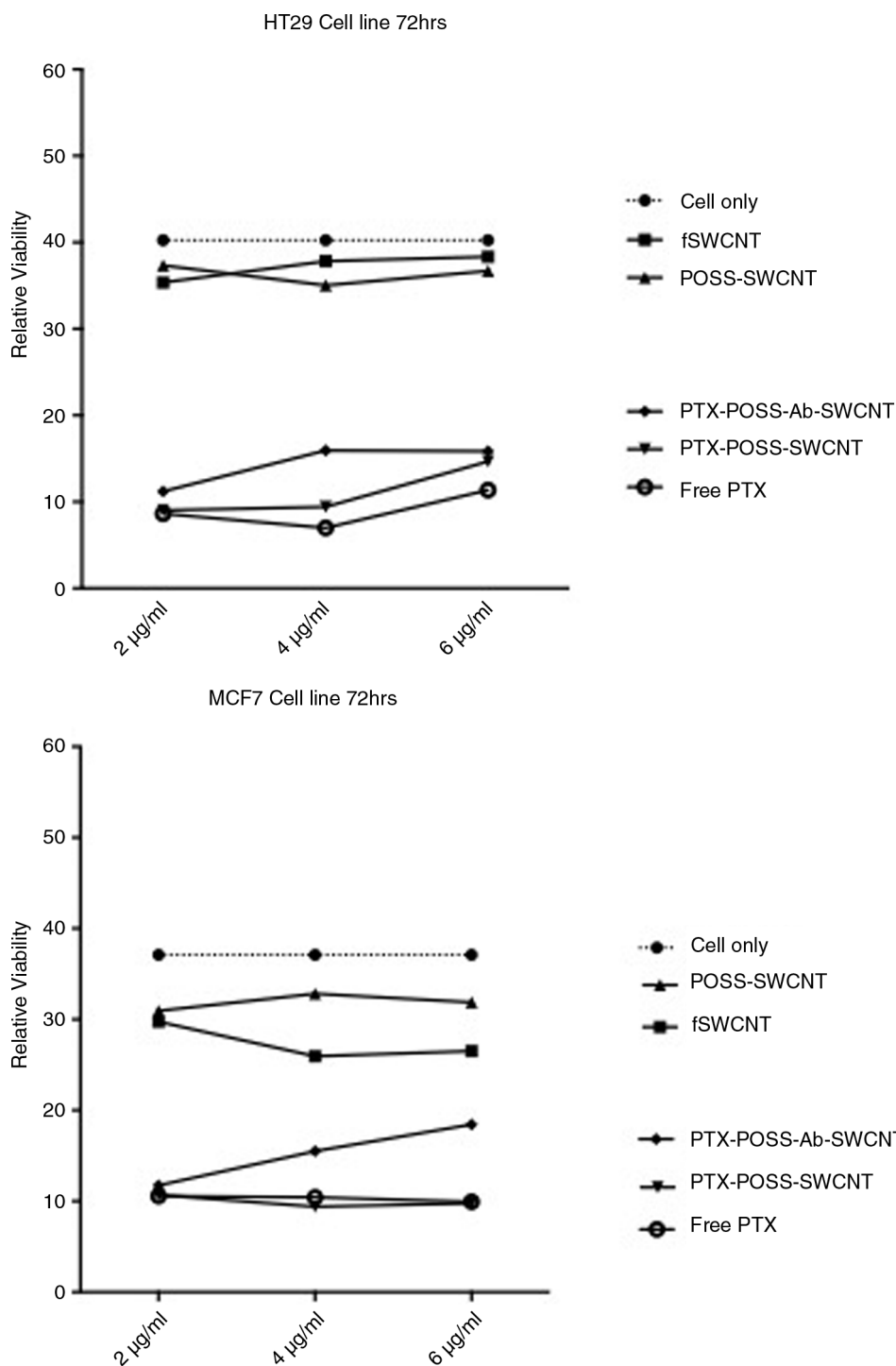

Fig. 5. AlamarBlue cell viability assessment 72 h after incubation in MCF-7 and HT-29 cells. Asterisk (*) indicates a statistically significant difference in cell viability between free PTX and PTX containing nanocomposites compared to controls (POSS–SWCNTs, −COOH functionalized SWCNTs and cell-only controls).

of commercial Taxol® after 3 days incubation when PTX concentrations were 10 and 20 µg/ml. Guo et al. showed that controllable drug release from a biodegradable coating of a PTX containing stent was achieved using nanostructured hybrid polyurethanes (POSS TPUs) (48), featuring alternating multiblock structures formed by nanostructured hard segments of polyhedral oligosilsesquioxane (POSS) thermoplastic polyurethanes and biodegradable soft segments of a polylactide/caprolactone copolymer (P(DLLA-*co*-CL)) incorporating PEG covalently. POSS aggregated to form crystals serving as physical crosslinks on the nanometre scale, while the soft segments were designed to modulate the drug release rate from the POSS TPU, with 90% of the drug releasing from within half a day to about 90 days PTX. Their study showed that the POSS TPUs allow a drug release rate that is effectively manipulated through variation in polymer glass transition temperature (T_g), degradation rate, and thickness increment rate (48). They went on to investigate the morphology, miscibility, and specific interactions of PTX with POSS TPUs (49). They found that PTX is amorphous in all proportions in the PTX/POSS TPU blends. PTX exhibited good miscibility in all PTX/POSS TPU blends. In addition, it also served as an antiplasticizer by increasing the blend T_g from that of the polymer to amorphous PTX. The glass transition breadth of the blends increased significantly only for drug concentrations higher than 50 wt% for most of the POSS TPUs, suggesting some spatial heterogeneity at these high concentrations. On the other hand, enhanced phase separation between the POSS hard segments and the TPU soft segments upon drug incorporation was also noted.

In general, the addition of a nanoparticle or polymeric molecule such as PEG on the surface of nanoparticles is
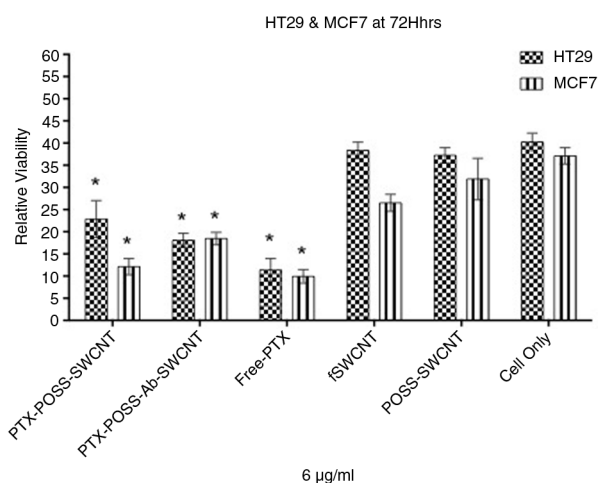
HT29 Cell line 72hrs

MCF7 Cell line 72hrs

Fig. 6. MCF-7 and HT-29 AlamarBlue® cell viability assessment with three different concentrations of the nanocomposites and controls after 72 h incubation. Increased cell death was observed with PTX–POSS–SWCNTs, PTX–POSS–Ab–SWCNTs, and free PTX compared to f-SWCNTs, POSS–SWCNTs, and cell-only controls ($p < 0.05$). There was no statistically significant difference in cytotoxicity between PTX-containing nanocomposites and free PTX.

required to avoid mononuclear phagocyte system uptake in the reticuloendothelial system (RES) organs (50, 51). Stabilization of nanoparticles can induce shifts in biodistribution, which may offer new opportunities for site-specific targeting. Compared to PEG and other polymers,

SWCNT–PTX exhibits short finite lengths (20–300 nm, mean ∼ 100 nm), a factor that favours long blood circulation since the average length of the nanotubes exceeds the threshold for renal clearance (52). Pharmacokinetics of materials with long blood circulation times are typically

desired for a drug delivery vehicle for tumour treatment (53) to mediate high tumour accumulation from the circulating blood through EPR effects. Note that our method of drug delivery by POSS-conjugated SWCNTs should be readily applicable to a wide range of hydrophobic or water-insoluble drugs. This could lead to a general drug delivery strategy for potent but water-insoluble molecules. The SWCNT–POSS–PTX-based nanocomposites were shown to be as cytotoxic as free PTX, with a late-onset effect compared to free PTX. At 24 h, free PTX was more effective against HT-29 cancer cells, but this effect was not evident at 48 and 72 h incubation. This is suggestive of a slow-release profile of PTX from the nanocomposites. This is an important finding as gradual and prolonged release of chemotherapeutic agents from nanocomposites may decrease their systemic toxicity and improve their blood circulation time. We used octa-ammonium POSS to render SWCNTs more biocompatible and water dispersable. As the POSS molecules have better reactivity and solubility, functionalization of POSS molecule with CNTs can substantially enhance the solubility and processability of the nanocomposite. Experiments have not shown any significant difference in cell viability, adhesion, and proliferation between POSS nanocomposites and standard cell culture plates. Evidence of biocompatibility and amphiphilic properties of POSS nanocomposites has already prompted researchers to patent these for use at the vascular interface such as stents (54, 55).

For the characterization of the nanocomposites, we used TEM, FTIR, and UV–Vis–NIR. An FTIR spectrometer measures the absorption spectrum of the sample, which corresponds to different functional groups. This technique is useful for detecting the presence of specific chemical groups on samples. AlamarBlue® Assay was used for cell viability assessment.

Our experiments showed that POSS–SWCNTs nanocomposites were not associated with decreased cell viability compared to cell-alone controls even after 72 h incubation. −COOH functionalized SWCNTs, on the other hand, were associated with increased MCF-7 cell death after 72 h incubation. This suggests increased biocompatibility of SWCNTs, when conjugated to POSS molecules. Our experiment did not involve any controls to show the effect of the nanocomposites on non-cancerous cells. In addition, we did not use any antibody only controls, assuming that their effect on cell viability would be negligible. These are the limitations of our study. Unlike the SWCNT carriers, which are excreted gradually in weeks or even months (56), the dissociated PTX drug molecules can be rapidly excreted via both faeces and urine without causing noticeable toxicity. The chemical composition (purely carbon) of CNTs is among the safest in the inorganic nanomaterials, many of which such as QD have heavy metal compositions. The unique structure and tuneable length provide an ideal platform to investigate

size and shape effects *in vivo*. Lastly, unlike the conventional organic drug carriers, the intrinsic spectroscopic properties of nanotubes including Raman and photoluminescence can provide valuable means of tracking, detecting, and imaging to understand the *in vivo* behaviour and drug delivery efficacy. Taken together, CNTs are promising materials for potential multimodality cancer therapy and imaging.

In this study, we report the successful use of CNT POSS nanocomposites as drug carriers to achieve *in vitro* efficacy. This opens up further exploration of biomedical applications of novel carbon nanomaterials with animals for potential clinical translation in the future. The treatment efficacy of SWCNT-based drug delivery vehicles could be further improved by optimization of the surface chemistry and size of nanotubes, as well as the positioning of drug molecules for desired pharmacokinetics.

In this study, we could not demonstrate any additional cytotoxic effect of antibody-conjugated nanocomposites, compared to non-targeting nanocomposites. A possible explanation for this observation may be that this was an *in vitro* study, and the effects of targeting would be evident in systemic *in vivo* administration of the nanocomposites. Chemotherapeutic agents are shown to be extremely effective *in vitro*. The toxic effects on healthy tissues associated with high-dose systemic administration limit their efficacy *in vivo*.

Due to the three-dimensional structure of PTX in which aromatic rings are not in a plane, there is not a strong non-covalent or π–π interaction between PTX and CNTs. The covalent linkage of CNTs and PTX provides an advantage for stabilization to avoid drug dissociation during the delivery process. We suggest that SWCNTs as nanomaterials have the potential to be utilized as drug carrier vehicles for cancer drug delivery and therapy. Previous reports have supported the release of covalently conjugated PTX from SWCNT. Liu et al. investigated the biodistribution of SWCNTs injected as PTX–SWCNT conjugates into mice by utilizing their intrinsic Raman scattering properties and observed high uptake of SWCNTs in the RES organs (22). PTX was released rapidly from SWCNT carriers in various organs and tissues after in vivo cleavage of the ester bond between SWCNT and PTX, likely by carboxylesterases (57, 58). Confocal fluorescence images indicated the endocytosis mechanism of the PTX–SWCNT uptake by cells (22). The treatment effect was further confirmed by tumour staining that revealed significant apoptotic cells and few proliferation active cells in the PTX–SWCNT treated tumour. They observed higher tumour suppression efficacy of PTX–SWCNT compared to free PTX and non-covalently conjugated PEG-PTX due to the up to 10-fold higher tumour uptake of PTX afforded by SWCNT carriers. Prolonged blood circulation and EPR effects are likely to be responsible for these effects. Pharmacokinetics of materials with long blood circulation times are typically

desired for a drug delivery vehicle for tumour treatment. High RES uptake is known for nanomaterials in general (14). The high uptake of PTX–SWNT in RES organs such as liver and spleen could be a cause of concern in terms of toxicity to these organs. Additionally, nanoliposomal delivery of cancer drugs has the benefits of prolonging drug in tissue residence during cancer therapy (59–61). Therefore, nanoparticles provide the opportunities for improving cancer therapeutics by being conjugated with cancer drugs (62).

We predict that CNTs will play an important role in the development of novel technologies for the treatment of cancer, as well as a wide range of other diseases. However, a significant number of questions remain to be addressed. In the future, the critical properties of CNTs that determine their biological activity should be defined, including chemical functionalization and effect of length and diameter. The potential toxicity of CNTs has been investigated and requires further characterization, although current evidence suggests that they are a safe option compared to other nanomaterials, such as QD, or other drug carriers, such as viral vectors. Further research and development in this field will undoubtedly lead to great clinical benefits to patients in the future.

In conclusion, we have synthesized a novel covalent linkage of SWCNT and PTX, which can deliver PTX to cancer cells. More importantly, the covalent bonding of SWCNT–PTX still preserves its anticancer activities. The combination of POSS, SWCNT, and PTX as a conjugate can be a novel strategy for cancer drug delivery and therapy.

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*Alexander M. Seifalian
UCL Centre for Nanotechnology & Regenerative Medicine
University College London
London WC1E 6BT, UK
Email: a.seifalian@gmail.com