Biomaterials 35 (2014) 4729–4738

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Aqueous cationic, anionic and non-ionic multi-walled carbon nanotubes, functionalised with minimal framework damage, for biomedical application

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ARTICLE INFO

Article history:
Received 6 January 2014
Accepted 4 February 2014
Available online 14 March 2014

Keywords:
Multiwalled carbon nanotubes
Thermal chemical functionalisation
Integrity structure
Surface charge
Cytotoxicity
Cell uptake

ABSTRACT

The use of a thermochemical grafting approach provides a versatile means to functionalise as-synthesised, bulk multi-walled carbon nanotubes (MWNTs) without altering their inherent structure. The associated retention of properties is desirable for a wide range of commercial applications, including for drug delivery and medical purposes; it is also pertinent to studies of intrinsic toxicology. A systematic series of water-compatible MWNTs, with diameter around 12 nm have been prepared, to provide structurally-equivalent samples predominantly stabilised by anionic, cationic, or non-ionic groups. The surface charge of MWNTs was controlled by varying the grafting reagents and subsequent post-functionalisation modifications. The degree of grafting was established by thermal analysis (TGA). High resolution transmission electron microscope (HRTEM) and Raman measurements confirmed that the structural framework of the MWNTs was unaffected by the thermochemical treatment, in contrast to a conventional acid-oxidised control which was severely damaged. The effectiveness of the surface modification was demonstrated by significantly improved solubility and stability in both water and cell culture medium, and further quantified by zeta-potential analysis. The grafted MWNTs exhibited relatively low bioreactivity on transformed human alveolar epithelial type 1-like cells (TT1) following 24 h exposure as demonstrated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and lactate dehydrogenase release (LDH) assays. The exposure of TT1 cells to MWNTs suppressed the release of the inflammatory mediators, interleukin 6 (IL-6) and interleukin 8 (IL-8). TEM cell uptake studies indicated efficient cellular entry of MWNTs into TT1 cells, via a range of mechanisms. Cationic MWNTs showed a more substantial interaction with TT1 cell membranes than anionic MWNTs, demonstrating a surface charge effect on cell uptake.

1. Introduction

The biological interactions of carbon nanotubes (CNTs) are important for fundamental scientific studies [1,2], biomedical applications such as photothermal therapy [3,4], drug delivery [5,6] and bioimaging [7,8], and in order to understand their potential toxicities [5,9,10]. The poor solubility and lack of control over the physicochemical properties of CNTs have created significant obstacles to understanding their interactions with cells. Delivery of individualised CNTs either *in vitro* in cell medium or *in vivo* is rare, particularly for toxicology studies, in which agglomerated or even non-aqueous dispersions are commonly used. Surfactants, such as Triton X-100 [5,6,11] and sodium dodecyl sulphate (SDS) [7,8,12], can be used to stabilise aqueous dispersions but use is limited by surfactant toxicity which can cause confounding effects [9,10,13] and do not relate to many real life situations. In addition, surfactant dispersions are meta-stable, with a surface chemistry that evolves over time, and are generally unsuitable for *in vivo* use [14]. Where modified, water-stabilised CNTs have been used, the

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http://dx.doi.org/10.1016/j.biomaterials.2014.02.002
0142-9612/

chemistry or dispersion processes introduce damage or dimensional change [15,16], limiting controlled comparisons. Improved protocols are needed to produce water compatible CNTs with well-defined dimensions whilst minimising changes to their intrinsic properties. Furthermore, surface modification of MWNTs can provide a product with enhanced and desirable properties for medicinal and other purposes. In addition, reasonably large quantities are required for consistent sets of *in vivo* and *in vitro* experiments, as well as a wide range of other CNT applications [17,18] in which aqueous processing would be advantageous.

One of the most common approaches for preparing water dispersions of CNTs relies on oxidation using strong acids, particularly mixtures of HNO_3 and H_2SO_4 [19,20]. This covalent functionalisation method produces good aqueous dispersions [20], and provides a high degree of functionalisation with −COOH groups suitable for further modification [21,22]. However, CNT walls are inevitably etched, the length is significantly reduced [23,24], and the sample is contaminated with oxidation reaction debris, also known as carboxylated carbonaceous fragments (CCFs), which are difficult to completely remove [25–27], and may contribute to cytotoxic effects [28,29]. Although the cytotoxicity of the oxidation debris is not yet fully understood, it has been shown that these CCFs can significantly alter the physicochemical properties and biological interactions of the acid-oxidised CNTs [25,26,30,31]. Further, the oxidation reaction also introduces a wide range of different, oxygen-containing functional groups, obscuring the nature of the underlying CNTs and generating an ill-defined generally acidic surface. Derivitising these surface functional groups (or, in some cases, unremoved debris) to form other chemical functionalities does not counter the remaining fundamental drawbacks [32]. Other chemical methods have also been explored [33,34], but a versatile, scalable method for varying surface character independently of other characteristics is still needed. The recent development of a solvent-free thermochemical grafting approach offers advantages in the preparation of large quantities of clean, functionalised MWNTs with minimal framework damage [35]. The approach takes advantage of existing defective groups on the MWNT surface, remaining from the synthesis process, which decompose at high temperatures and generate free radicals, allowing covalent grafting of a range of monomers [35]. The intrinsic framework of the MWNTs is preserved and there is no production of debris or use of corrosive solutions. The approach is readily scalable as it is compatible with common chemical vapour deposition (CVD) capital infrastructure and can be operated to avoid time-consuming filtration steps. Here, this protocol was developed to produce water-compatible MWNTs with cationic, anionic or non-ionic character for biological studies. The vast majority of nanotubes produced commercially are multi-walled structures prepared by CVD; these materials are produced in significant quantities, by different companies, but with similar characteristics [36], and are most relevant to near term application and occupational hazards.

The effects of MWNTs on lung cells are particularly relevant to discovering possible nanotoxicological pathways, since inhalation is considered to be one of the greatest exposure risks [37,38]. Similar to other thin materials, regardless of length, MWNTs with diameters smaller than 1 μm are respirable and may be able to travel deeply into the lung alveolar region, where the thin gas–blood barrier (in some places <0.5 μm deep) is located to allow gas exchange [39,40]. Alveolar macrophages are the first line of defence against particulate material which deposit in the alveoli. Particles are taken up into macrophages through phagocytosis, and subsequently cleared out of the lung via the mucociliary escalator and lymphatic system. Particles that are not phagocytosed by macrophages will interact with the alveolar epithelium [40,41], which comprises a monolayer of alveolar type I (AT1) and alveolar type II (AT2) epithelial cells [42]. AT1 cells, which cover >95% of the alveolar surface area, are large squamous cells that facilitate gas exchange [43]. There is some concern that the interaction of high aspect ratio particles with alveolar epithelial cells could induce similar pathologies to those observed with high aspect ratio amphibole asbestos, including epithelial cell necrosis or apoptosis, release of pro-inflammatory cytokines, which could compromise the integrity of the epithelium, and increase the chance of particle translocation across the gas-blood barrier to the interstitium and systemic circulation [44].

This paper describes the generation and properties of a panel of surface functionalised MWNTs with anionic, cationic or non-ionic groups and high water solubility, relevant to a wide range of applications. Here, their bioreactivity with the human lung epithelium is studied *in vitro*. The effects on transformed human alveolar type 1-like epithelial cells (TT1) cell viability and mediator release have been assessed and the extent of uptake studied by TEM.

2. Materials and methods

2.1. Carbon nanotubes and chemicals

MWNTs (diameter 12.1 nm, s.d. 3.7 nm), synthesised by CVD, were obtained from Arkema SA (Lacq-Mourenx, France). Methyl methacrylate (MMA, >98.5%), 4-vinyl pyridine (4-VP, 95%), poly(ethylene glycol) methacrylate (PEGMA, average M_n = 530), 1-iodododecane (IDD, 98%), iodomethane (IMe, ≥ 99%), and lithium hydroxide (LiOH, 98%) were purchased from Sigma–Aldrich for MWNTs functionalisation. Before use, all chemicals were passed through a chromatographic column consisting of neutral and basic aluminium oxide powders (aluminium oxide 90 (0.063–0.200 mm), activity stage I for column chromatography, Merck Millipore, Germany) and further degassed by bubbling N_2 gas for 30 min, in order to remove radical inhibitors and oxygen.

2.2. Functionalisation of MWNTs

2.2.1. Thermochemical grafting

The thermal activation process was carried out in a custom-made 30 mm diameter quartz tube attached to a sample flask, and the whole setup was connected to a vacuum system. In a typical experiment, 100 mg MWNTs were heated to 1000 °C at a constant ramping rate of 10 °C/min under vacuum ($\sim 5 \times 10^{-4}$ mbar), in a three-zone tube furnace (PTF 12/38/500, Lenton Ltd, UK) and held at the activation temperature for 2 h. After the activation step, the quartz tube was slowly removed from the heating zone and allowed to cool to room temperature under vacuum. The MWNTs were then transferred to the connected round bottom flask by gravity. 8 mL of the reactant was then injected into the flask containing the thermally-activated MWNTs. The reaction mixture was stirred at room temperature overnight. The unreacted reactant was removed via filtration through a 0.45 μm pore size polytetrafluoroethylene (PTFE) membrane (Whatman, UK) under vacuum. The product was thoroughly washed with 3 × 90 mL of washing solvent, then dispersed in 90 mL of solvent and bath sonicated (USC300T, 45 kHz, 80W, VWR International, USA) for 15 min. The filtration–sonication cycle was repeated three times in order to remove any physically absorbed reactants. The functionalised MWNTs are named by the abbreviation of the grafted polymer: e.g. P(MMA)-MWNT. The other sample codes can be found in Table 1.

2.2.2. Synthesis of P(M4-VP)-MWNT

P(4-VP)-MWNTs (20 mg) were dispersed in 10 mL of methanol (99.8%, Sigma–Aldrich) by bath sonication for 5 min; IMe (3.12 mL, 50.0 mmol) was added drop-wise, and the reaction mixture was heated to 60 °C overnight under N_2 atmosphere [45]. Afterwards, the mixture was cooled to room temperature and filtered through a 0.45 μm PTFE membrane. The MWNTs were washed with 3 × 30 mL of ethanol, then dispersed in 30 mL of ethanol and bath sonicated for 15 min. The filtration–sonication cycle was repeated three times in order to remove any physically absorbed reactants.

2.2.3. Synthesis of P(MAA)-MWNT

LiOH (40 mg) was dissolved in 20 mL 10:1 v/v THF/water cosolvent before adding 20 mg of P(MMA)-MWNT. The reaction mixture was bath sonicated for 5 min to obtain a good dispersion, and then stirred at room temperature overnight. Subsequently, 37% hydrochloric acid (HCl, AnalaR grade, BDH) was added drop-wise until the pH value of the solution reached pH 2 [46]. The mixture was stirred for another 12 h, then filtered on a 0.45 μm PTFE membrane, and washed with water (3 × 30 mL). The MWNT residue was dispersed in 30 mL of water by bath sonication for 15 min. The filtration–sonication cycle was repeated three times in order to remove any remaining salt and acid.