Aggregation behaviour of carbon nanotubes in aqueous and physiological media and its influence on toxicity

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Abstract: Extensive use of carbon nanotubes (CNTs) in industry and in the consumer market including drug delivery will increase the likelihood of human exposure and the contamination of the ecosystems. The aggregation state of CNTs has a strong influence on their fate and transport in the environment, as well as their bioavailability. Studies on their colloidal behaviour suggest that they can be relatively stable in aqueous and biological medium, and the presence of surfactants and macromolecules may enhance their dispersibility and persistence. This paper is a review of the aggregation behaviour of CNTs as well as their functional analogues, and the possible role of the dispersal state on toxicity.

Keywords: carbon nanotubes; CNTs; dispersion; aggregation; physiological media; water; toxicity.

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1 Introduction

Carbon nanotubes (CNTs) are characterised by some highly desirable mechanical, thermal and electrical properties, which make them attractive for a wide range of applications ranging from field emission to reinforcements in nanocomposites (Kong et al., 2000; Murugesan et al., 2006; Petrov et al., 2004; Yun et al., 2006). They are essentially graphene sheets that have been seamlessly rolled into cylindrical tubes. They can be single-walled (SWCNTs), double-walled (DWCNTs) or multi-walled (MWCNTs) depending on the number of concentric rings (Iijima, 1991, 2002; Zhou et al., 2002). The applicability of CNTs is often limited by their inherent incompatibility with solvents and polymers.

With largely defect-free sidewalls CNTs are rather inert to chemical attack, tend to be hydrophobic, and are difficult to disperse or dissolve in water and other organic solvents. Some level of functionalisation is therefore required prior to their utilisation in real-world applications. This has mandated a plethora of studies on the solubilisation of CNTs using diverse techniques (Tasis et al., 2006). The three main approaches that have been used to solubilise CNTs are surfactant-based solubilisation (Islam et al., 2003), non-covalent wrapping/adsorption, where soluble polymers have been wrapped on the CNTs (Lin et al., 2003), and covalent functionalisation involving chemical modification of the CNT surface (Chen and Mitra, 2008; Huang et al., 2002; Mickelson et al., 1999; Pompeo and Resasco, 2002; Wang et al., 2005a, 2005b). The latter techniques include covalent tethering, such as, fluorination (Mickelson et al., 1999), amidation (Huang et al., 2002), glucosamine attachment (Pompeo and Resasco, 2002), and sidewall carboxylic acid functionalisation (Peng et al., 2003).

Extensive use of CNTs in industry and the consumer market will increase the likelihood of CNT exposure to the natural environment. CNTs can be released into the environment via wastewater discharge as point source emissions from manufacturing industries (Wiesner et al., 2006). Upon release, they will interact with the aquatic environment and biological species. Depending on the interplay between electrostatic and van der Waals interactions, they will aggregate (Thess et al., 1996). The aggregation state of CNTs has a strong influence on their fate and transport in the environment. Therefore, understanding the factors governing the aggregation behaviour of CNTs is key to evaluating their potential interaction with biological species. Literature on CNT aggregation has mostly been centred on enhancement of their aqueous solubility, either by dispersing CNTs by surfactant (Jiang et al., 2003; Lisunova et al., 2006), polymer adsorption (Jung et al., 2004), or by introducing oxygen containing functional groups on the surface through acid treatment (Chen and Mitra, 2008; Peng et al., 2003; Wang et al., 2005a, 2005b). However in recent years the number of publications studying the aggregation behaviour of CNTs in the presence of electrolytes has increased significantly, where time-resolved dynamic light scattering (TRDLS), Raman spectroscopy, zeta-potential measurements, and UV-Visible spectroscopy have been used to investigate the aggregation kinetics of CNTs in aqueous and physiological media (Peng et al., 2009; Smith et al., 2009).

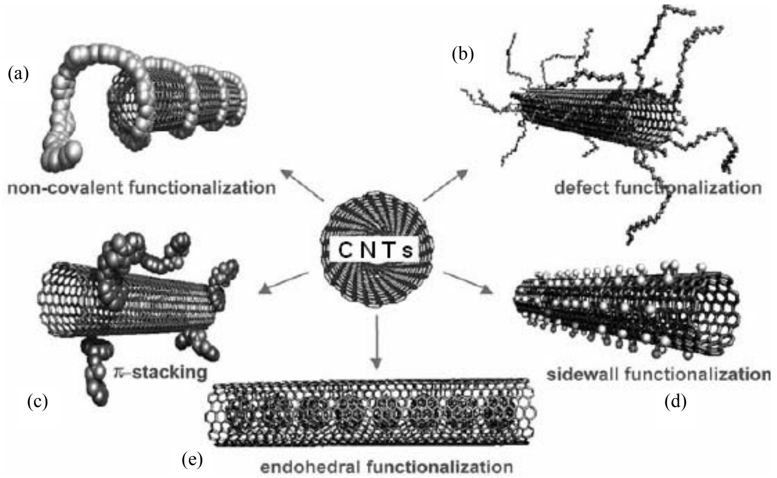
The dispersal state of CNTs is one of the most important physicochemical characteristics that need critical attention during cytotoxicological studies because this determines their bioavailability and therefore interactions at the nano-bio interface. Therefore understanding the mechanisms involved in controlling the state of CNT agglomeration in physiological media is very important to assess the toxic effects expressed by aggregated tubes as opposed to the dispersed ones. Since there is a high propensity for CNTs to aggregate due to electrostatic attractions, efforts have been aimed at achieving well dispersed CNT formulations for toxicological analysis using surfactants and dispersing agents. Some dispersants such as fetal bovine serum (FBS), tween and bronchoalveolar lavage (BAL) of rodents have been successfully used in dispersing CNTs in tissue culture media (Jia et al., 2005; Muller et al., 2005; Porter et al., 2008; Sager et al., 2007; Shvedova et al., 2003; Warheit et al., 2004). Synergistic effects of proteins and surfactants in CNT dispersion have also been reported (Chen et al., 2001; Li et al., 2004). The effect of the dispersal state of CNTs on their cytotoxicity has also been reported with varied impacts on cell activity and proliferation observed (Mercer et al., 2008; Shvedova et al., 2005; Wick et al., 2007).

The objective of this paper is to discuss the aggregation behaviour of CNTs under various environmental and biological conditions. Of particular interest is the influence of surface functionalisation and CNT dimensions. Dispersibility in physiological media as well as the influence of surface modification and dispersal state on toxicological studies will also be discussed.

2 CNT functionalisation

CNTs in their pristine form are generally chemically stable and insoluble in water or organic solvents. This limits their applicability in systems that require the preparation of homogenous mixtures of CNTs with different organic, inorganic, and polymeric materials. Some level of functionalisation is therefore necessary where certain molecules or functional groups are physically or chemically attached to their sidewalls without significantly changing their physical/electronic properties. A wide range of molecules and functional groups such as lipids, proteins, biotins, DNA, poly-aminobenzoic sulfonic acid (PABS), polyimide, polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), etc. (Addo Ntim et al., 2011; Chen et al., 2001; Li et al., 2004; O'Connell et al., 2001) have been attached to CNT surfaces. Several routes to CNT functionalisation (Figure 1) have been reported (Hirsch and Vostrowsky, 2005); however non-covalent wrapping/adsorption and covalent tethering are the two main routes which have been extensively investigated. Covalent functionalisation is based on covalent linkage of functional entities onto the nanotube's carbon scaffold, either at the end caps of the tubes or sidewalls. This can involve either a direct covalent functionalisation where a change in hybridisation from sp^2 to sp^3 and a simultaneous loss of conjugation occurs, or defect functionalisation where chemical transformations occurs at defect sites already present at the open ends on the sidewalls, or pentagon and heptagon irregularities in the graphene framework. Non-covalent functionalisation on the other hand involves supramolecular complexation using various adsorption forces, such as van der Waals' and π-stacking interactions.

Figure 1 Routes of CNT functionalisation, (a) Non-covalent exohedral functionalisation with polymers (b) Defect-site functionalisation (c) Non-covalent exohedral functionalisation with molecules through p-stacking (d) Sidewall functionalisation (e) Endohedral functionalisation of C60 into the lumen of SWCNT



Source: Hirsch and Vostrowsky (2005)

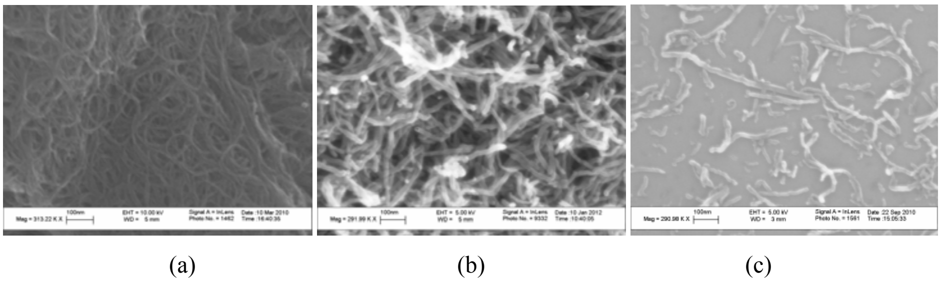
2.1 Physicochemical characterisation

CNTs from several different vendors and manufacturers have been used in the studies of aggregation behaviour. Some of the studies did not mention the source of their CNTs and others used CNTs produced in the laboratory of collaborators (Chen et al., 2004; Peng et al., 2009; Shieh et al., 2007). Some typical examples are given below. Niyogi et al. (2007) and Sano et al. (2001), studied SWCNTs from Tubes@Rice with outer diameter (OD) of 1.2 nm. Jiang et al. (2003) used CNTs from Shenzhen Nanotech Port Ltd. Co. (Shenzhen, China), the nature of CNTs and their dimensions were however not specified. Since the effect of size was not considered an important parameter, Sinani et al. (2005) did not report the dimensions of the SWCNTs they studied from Nanocyl (Namur, Belgium), Carbon Nanotechnology Inc. (Houston, TX) and Southwest Nanotechnologies (Norman, OK); and the MWCNTs from Nano-Lab (Boston, MA). Southwest Nanotechnologies (Norman, OK) and Nano-Lab (Boston, MA) also supplied the SWeNT (OD: 0.8 ± 0.1 nm) and SWCNT (OD: ~1.5 nm; length: 1–5 µm) respectively, studied by Heister et al. (2010) MWNTs of different dimensions used in their study were obtained from sigma Aldrich (ODs: 10–20 nm; length: 0.5–200 µm, and OD: 110–170 nm; length: 5–9 µm) and DWNTs synthesised at CIRIMAT-LCMIE, Universite' Paul Sabatier in Toulouse, France. Saleh et al. (2008) and Smith et al. (2009) studied MWCNTs purchased from Nanotech Labs Inc (Yadkinville, NC) with dimensions (ODs: 20–40 nm; length: 50 µm) and (OD: 15 ± 5 nm; length: 5–20 µm) respectively. Cheaptubes Inc. (Brattleboro, VT) supplied the SWCNTs used by Saleh et al. (2010) (OD: 0.8–1.6 nm; length: 5–30 µm) and the MWCNTs studied by Wang et al. (2010) (OD: 20–30 nm; length: 10–30 µm) and Addo Ntim et al. (2011) (OD: 20–30 nm; length: 10–30 µm) (Addo Ntim et al., 2011; Saleh et al., 2010; Wang et al., 2010). The purity of the CNTs studies studied ranged from (45–95)% with the composition of the impurities being predominantly residual metal catalysts and non-tubular carbon. Differences in manufacture methods lead to different physicochemical characteristics such as length, shape and functionalisation, which can influence dispersibility and toxicity. However, at this time there are insufficient studies to provide a clear systematic analysis of such influences.

The key physicochemical properties of the CNT samples studied are often determined by electron microscopy (SEM and TEM), atomic force microscopy (AFM), thermogravimetric analysis (TGA), Raman spectroscopy and Fourier transform infrared spectroscopy (FTIR). Saleh et al. (2008, 2010) used a high resolution transmission electron microscope to image MWCNT and SWCNT samples to determine the diameter and length distribution. The TEM data showed significant debundling and shortening of the MWCNTs after sonication and dark spherical features on the image which they presumed to be catalyst particles. Isolated single MWCNTs were also observed which they attributed to a high degree of dispersion due to sonication treatment. The diameter and length distribution reported based on the TEM analysis was not in agreement with the data supplied by the MWCNT manufacturer (Saleh et al., 2008). A similar tube shortening with sonication was observed from the TEM images of the SWCNTs (Saleh et al., 2010). It was also observed that the majority of the tubes were SWCNTs, with few larger diameter tubes, most likely double-walled or multi-walled (Saleh et al., 2010). Several other studies have used high resolution TEM to study the morphology of CNTs mainly confirming tube structure before and after processing (Heister et al., 2010; Jiang et al., 2003; Shieh et al., 2007).

In instances where TEM was not used SEM was used to characterise tube morphology (Addo Ntim et al., 2011; Desai et al., 2012; Jiang et al., 2003; Shieh et al., 2007; Wang et al., 2010). Addo Ntim et al. (2011) used Scanning Electron Microscope equipped with an energy-dispersive X-ray analyser (EDS) to demonstrate that MWCNTs after acid functionalisation retained their tube structure with minimal visible damage. The same system was used by Wang et al. (2010) and Desai et al. (2012). The EDS system attached to the SEM was used to analyse the elemental composition in the as-prepared and functionalised MWCNTs (Addo Ntim et al., 2011; Desai et al., 2012; Wang et al., 2010). Figure 2 shows CNTs imaged with scanning electron microscopy.

Figure 2 CNTs imaged with scanning electron microscopy, (a) Single walled CNTs (b) Multi-walled CNTs (c) Short MWCNTs


(a) (b) (c)

In most of the studies involving functionalised CNTs, FTIR was used to confirm the presence of functional groups (Addo Ntim et al., 2011; Desai et al., 2012; Shieh et al., 2007; Wang et al., 2010). The carboxylic (C=O) stretching frequency in oxidised MWCNTs is often reported to occur around 1,700 cm^{-1} with the O-H vibration occurring around 3,400 cm^{-1} (Addo Ntim et al., 2011; Desai et al., 2012; Shieh et al., 2007; Wang et al., 2010). PVP wrapping was confirmed by the amide stretching vibration around 1,645 cm^{-1} (Addo Ntim et al., 2011). The presence of octadecylamine (ODA) on the surface of MWCNTs was confirmed by the amide stretching vibration around 1,639 cm^{-1} and the stretching vibrations of the alkyl chain around 2,847 cm^{-1} and 2,921 cm^{-1} (Desai et al., 2012). The purity of CNTs studied, and in some instances percentage of functional groups present, are investigated using TGA. TGA is carried out either under an air or nitrogen flow at specified flow and heating rates. As-prepared MWCNTs often decompose between 500–600°C with SWCNTs decomposing around 450°C (Addo Ntim et al., 2011; Desai et al., 2012; Saleh et al., 2010). Addo Ntim et al. (2011) used TGA to determine the extent of carboxylation in oxidised MWCNTs as did Wang et al. (2010) and Desai et al. (2012) in addition the amount of PVP wrapping and ODA functionalisation were also determined using TGA.

Raman spectroscopy can be used to estimate SWCNT diameter and also to obtain information about its defect level. A typical Raman spectra of SWCNTs exhibits two active Raman signature modes, namely, the radial breathing mode (RBM, near 200 cm^{-1}) and a high-energy mode (HEM or ‘G’ band, near 1,600 cm^{-1}), and another electric resonance mode originating from double resonance called the ‘D’ band (near 1,300 cm^{-1}) (Thomsen and Terkildsen, 1975). The Raman shift frequency at the RBM mode is correlated with SWCNT diameter using semi-empirical relationships. Saleh et al. (2010) estimated the diameter of SWCNTs based on their Raman spectra to be 0.87–1.42 nm. They also reported that the defect density in SWCNTs estimated by the G/D ratio was lower in sonicated SWCNTs than in as-prepared SWCNTs, indicating an increase in defect density due to successive sonication of the SWCNT samples, consistent with their previous observation with MWCNTs (Saleh et al., 2008). Heister et al. (2010) used Raman spectra to confirm diameter of SWCNT and to demonstrate that NaOH washing of oxidised SWCNTs removes oxidative debris resulting in slightly lower D/G ratio indicating the removal of defective material from the sample.

3 Dispersibility of CNTs

3.1 CNT electrokinetic properties

A charged colloidal particle suspended in an electrolyte solution is surrounded by a cloud of counterions which contribute to a set of surface charges and counter charges called the electric double layer. This plays an essential role in various interfacial electrical phenomena on the particle surface and in the particle-particle interaction in the colloid suspension. The surface potential on colloid particles is generally difficult to measure. However, the potential near the particle surface, called the zeta (ζ) potential, is measurable. The ζ-potential provides useful information regarding the stability of colloidal suspensions in diverse applications including food preparations, agriculture, pharmaceuticals, paper industry, ceramics, paints, coatings, photographic emulsions, etc. Zeta potential has been used to study the stability of MWCNT dispersions and the influence of counterions on their aggregation behaviour (Addo Ntim et al., 2011; Peng et al., 2009). In some cases electrophoretic mobility (EPM) which is essentially the migration of charged colloidal particles through a solution under the influence of an applied electric field has been used instead of zeta potential (Saleh et al., 2008, 2010; Smith et al., 2009). Generally CNTs exhibit negative surface potential in aqueous systems (Addo Ntim et al., 2011; Jiang et al., 2003; Peng et al., 2009; Saleh et al., 2008, 2010; Smith et al., 2009). While the negative surface charge in functionalised CNTs is attributed to the presence of acidic groups introduced through acid treatment, the origin of surface charge is not fully understood for unfunctionalised carbon-based nanomaterials such as fullerenes and CNTs.

Jiang et al. (2003) reported that adsorption of sodium dodecyl sulphate (SDS) improved the zeta potential CNT dispersions at pHs below 8. This was however not observed at higher pH values where the zeta potential values were essentially the same for both dispersion systems (with and without SDS). This was attributed to the presence of acidic sites on the surface of the CNTs purified by acid treatment which can be dissociated in the alkaline solutions. It was demonstrated that the presence of SDS resulted in more negative zetapotential values (–40 mV) translating to better stabilised MWCNT dispersions, than was observed in the dispersions without SDS (–30 mV). This was true for the entire pH range studied (3–11). The effect of SDS was attributed to the electrostatic repulsion provided by adsorbed surfactants which stabilised the CNTs against van der Waals attraction (Vigolo et al., 2000). In a similar study by Saleh et al. (2008) the EPM of MWCNTs dispersions was reported to decrease, becoming less negative with increasing salt concentration. The presence of defects on the surface of CNTs in the form of pentagon and heptagon irregularities at their carbon scaffold and also incomplete carbon rings at the end termini make the sidewalls and tube-ends susceptible to oxidation to form carboxyl and hydroxyl functional groups (Hirsch and

Vostrowsky, 2007). These functional groups present on the CNT surface accounts for the strong dependence of EPM on solution pH. Saleh et al. (2008) observed that the presence of Suwannee River humic acid (SRHA) did not significantly affect the EPM of the MWCNT dispersions under the solution chemistries investigated. In another study involving SWCNTs in the presence of biomacromolecules and humic acid, Saleh et al. (2010) reported a similar decrease in EPM with increasing salts concentration as was observed for the MWCNTs (Saleh et al., 2008). The SWCNTs were observed to have a negative surface charge which the authors attributed to mechanochemistry (Saleh et al., 2010). The presence of biomacromolecules and humic acid did not significantly increase in the EPM values of the SWCNTs contrary to previous observations where adsorbed surfactants and polyelectrolytes reportedly enhanced the EPM of SWCNTs (Liu et al., 2007; White et al., 2007).

Smith et al. investigated the influence of pH on the EPM of the oxidised MWCNT in the presence of 64 mM NaCl. The EPM values decreased with increasing pH from pH 3 to 5 with the values remaining fairly stable above pH 6, independent of the electrolyte concentration (Smith et al., 2009). This was consistent with previously reported EPM measurements of oxidised CNTs (Esumi et al., 1996; Hu et al., 2005). The steady EPM at pH > 6 was however not explained. Addo Ntim et al. (2011) compared the zeta potential values of carboxylated and polymer wrapped MWCNTs in the presence of Na^+ and Mg^{2+} and observed that zeta potential become less negative with increasing salt concentration for both MWCNT derivatives. However the decrease was less prominent in the polymer wrapped system than the carboxylated system where electrolyte ions neutralised the negative surface charge result in decrease electrical double layer and therefore increase the propensity for the tubes to aggregated make the dispersion less stable. A similar observation was made by Peng et al. (2009) while investigating the effect of electrolytes on the stability of oxidised MWCNTs.

3.2 Aggregation behaviour in aqueous media and the effect of electrolytes

The aggregation behaviour of both as-prepared and functionalised CNTs in aqueous media have been studied, with particular emphasis on the influence of dissolved salts and pH. In some instances the effects of natural organic matter and biomacromolecules have also been investigated. Precipitation of CNTs under the influence of different salts often show dependence on the charge on the cations, and in general these nanoparticles have followed the well-established Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Derjaguin and Landau, 1993; Verwey and Overbeek, 1999). In aqueous systems, electrolyte ions are known to coagulate certain colloidal sols. It is well-known that the critical coagulation concentration (*ccc*), the minimum concentration of ions necessary to cause rapid coagulation of colloids follows the Schulze-Hardy rule

$$ccc \sim \left(\frac{1}{z}\right)^n$$

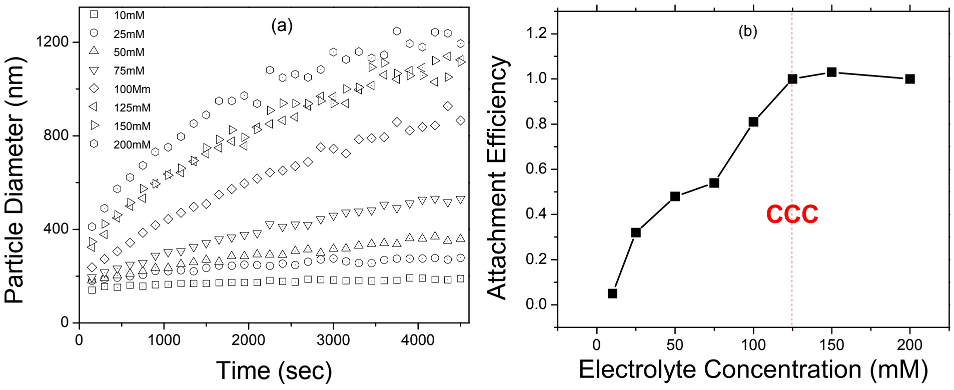
where z is the valence of the electrolyte counterions. Typically, n is 6 in three dimensions (3D) and 9 in two dimensions (Sano et al., 2000). According to the DLVO theory, the Schulze-Hardy rule results from interplay between van der Waals attraction and electric double-layer repulsion (Israelachvili, 2010).

Sano et al. (2001) were among the first group to investigate the colloidal nature of SWCNTs in electrolyte solutions particularly emphasising the dependence on the Schulze-Hardy rule. They studied the aggregation behaviour of 0.6 mg/mL dispersions of shortened, oxidised SWCNTs in the presence of Na^+, K^+, Mg^{2+}, Ca^{3+}, La^{3+} and Ce^{3+} by first dispersing the SWCNTs in an electrolyte solution by utrasonication and then measuring the UV absorbance of the supenantant after allowing the dispersions to sit undisturbed for 12 h at room temperature. The *ccc* values obtained for Na^+, K^+, Mg^{2+}, Ca^{3+}, La^{3+} and Ce^{3+} were 37, 26, 0.31, 0.20, 0.050 and 0.052 respectively. A clear inverse relationship between the *ccc* values and the counterion valence z was observed, and a plot of the double logarithm of the *ccc* values against valence z produced a straight line with a slope of –6 (Sano et al., 2001). It was therefore concluded that SWCNTs followed the 3D Schulze-Hardy rule.

Jiang et al. (2003) used a UV-visible spectrophotometric method to compare the stability of CNT dispersion made with and without SDS. CNT dispersions at 0.2 wt% were made with and without SDS (0.1 wt%) at pH 9 and the UV-visible absorbance of the dispersions was measured as a function of time over a period of 500 h. It was observed that in both dispersions, CNT concentration reduced with time, with the SDS stabilised dispersion showing better stability (Jiang et al., 2003). The optimal conditions necessary to make stable homogenous CNT dispersions were reported to be 0.5 wt% CNT and 2.0 wt% SDS. Jiang et al. also investigated the effect of pH on the zeta potential of the dispersions which is discussed in detail under the electrokinetic properties section of this paper. Aqueous dispersions of SWCNTs containing individual tubes can be achieved using SDS. Once intertube van der Waals attraction is overcome by intense sonication, free SDS adsorbs to SWCNT surfaces and creates a net surface density of negative charge, which prevents SWCNT reaggregation. Any change in the surface charge density or solubility of the surfactant, that eliminates the electrostatic repulsion between the nanotubes will cause SWCNTs to aggregate and coagulate (Tan and Resasco, 2005). Niyogi et al. (2007) demonstrated that by controlling the interplay between intermolecular and surface forces of SDS in water it was possible to engineer the resultant van de Waals attraction between SWCNTs to achieve selective aggregation. They studied the absorbance and emission spectra of SDS assisted SWCNT dispersions after the addition of NaCl and observed that the addition of Na^+ decreased the electrostatic repulsion between SDS molecules resulting in an increase in their aggregation number. Thus, manipulation of SDS equilibria results in loss of free SDS in solution through precipitation or further micellisation, causing a loss in SWCNT-bound in response. This causes in nanotube aggregation as electrostatic repulsion is reduced (Niyogi et al., 2007). A dependence on the DLVO theory of the SWCNT aggregation was observed where an increase in salt concentration resulted in a corresponding increase in CNT aggregation. The valence of counterions also affected the SWCNT aggregation according to the Schulze-Hardy rule.

Sinani et al. studied the stability of positively charged suspensions of SWCNTs and MWCNTs in water and/or mixed polar solvents, investigating the influence of CNT manufacturer on suspension stability. SWCNTs from three different vendors were studied (Sinani et al., 2005). Several block copolymers namely poly(N-cetyl-4-vinylpyridinium bromide-co-N-ethyl-4-vinylpyridinium bromide-co-4-vinylpyridine) (16 75/9), poly(vinylpyrro-lidone-co-allylamine), poly(N-ethyl-4-vinylpyridinium bromide-co-4-vinylpyridine) (94/6), poly(N-ethyl-4-vinylpyridinium bromide-co-4-vinylpyridine) (30/70), and poly-(N-dodecyl-4-vinylpyridinium bromide-co-N-ethyl-4-vinylpyridinium bromide-co-4-vinyl-pyridine (36/51/13) were examined as CNT dispersing agents. It was observed that only poly(N-cetyl-4-vinylpyridinium bromide-co-N-ethyl-4-vinylpyridinium bromide-co-4-vinylpyridine) (16/75/9) and poly-(N-dodecyl-4-vinylpyridinium bromide-co-N-ethyl-4-vinylpyridinium bromide-co-4-vinyl-pyridine (36/51/13) displayed efficient solubilisation of the CNTs. It was speculated that those two polymers could potentially be used as universal dispersing agents based on the fact that they successfully produced stable dispersions of both SWCNT and MWCNT and also the difference in CNT vendors did not affect the stability of the colloidal system.

Figure 3 (a) Hydrodynamic size as a function of time in the presence of electrolytes (b) Attachment efficiency as a function of electrolyte concentration (see online version for colours)



(a) (b)

Source: Addo Ntim et al. (2011)

Saleh et al. (2008) investigated the initial aggregation kinetics of as-prepared MWNCTs using TRDLS (Saleh et al., 2008). They evaluated the influence of pH, concentration of monovalent (NaCl) and divalent ($CaCl_2$ and $MgCl_2$) salts and the presence of natural organic matter (SRHA) on the aggregation kinetics of the MWCNTs. A TRDLS method was used to study the initial aggregation kinetics of MWCNT in the presence of electrolytes and natural organic matter where the hydrodynamic radius of the MWCNTs in the dispersion was measured as function of time over a period of 3 hours to obtain approximately 30% increase in the original hydrodynamic radius of the MWCNTs. Figure 3(a) shows a representative hydrodynamic radius/time profile generated from TRDLS analysis. The attachment efficiency of the MWCNTs was then determined based on the initial aggregation rate constant of MWNTs which is proportional to the initial rate of increase in the hydrodynamic radius, R_h, with time, t, and the inverse of MWNT concentration N_0 (Chen and Elimelech, 2007):

$$k \propto \frac{1}{N_o}\left(\frac{dR_h(t)}{dt}\right)_{t \to 0}$$

For MWCNT dispersions with the same nanotube concentration, the attachment efficiency, α, (which is also the inverse of the Fuchs stability ratio, W, commonly used in colloidal stability studies) is obtained by dividing the initial slope of the aggregation profile of a given solution chemistry by the initial slope obtained under favourable (fast) aggregation conditions:

$$\alpha = \frac{\left(\frac{dR_h(t)}{dt}\right)_{t\to 0}}{\left(\frac{dR_h(t)}{dt}\right)_{t\to 0,\, fast}}$$

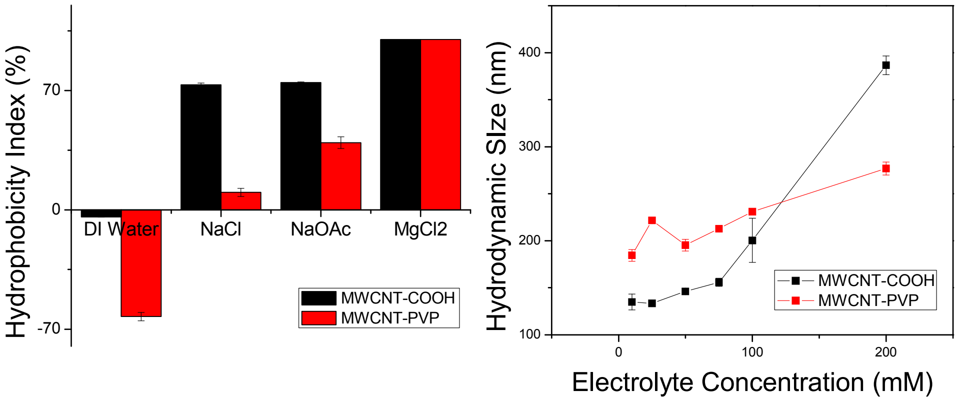
where the subscript ‘fast’ represents favourable solution conditions, under which rapid, diffusion-limited aggregation takes place. The *ccc* values are then obtained from the intersection of the slow and fast aggregation regimes of the attachment efficiency/electrolyte concentration profile as shown in Figure 3(b).

Saleh et al. (2008) observed an increase in MWCNT aggregation with increasing salt concentration and counterion valence consistent with the DLVO theory and the Schulze-Hardy rule. The *ccc* values obtained for the monovalent NaCl was 25 mM, slightly lower than the value obtained by Sano et al. (2001) for SWCNT (37 mM). For the divalent salts however the *ccc* values (2.6 mM Ca^{2+} and 1.5 mM Mg^{2+}) were higher than those for SWCNT (0.2 mM Ca^{2+} and 0.3 mM Mg^{2+}) (Sano et al., 2001). The attachment efficiencies of the MWCNTs were observed to significantly decrease in the presence of SRHA for aqueous solutions containing monovalent and divalent salts. This was attributed to non-DLVO, steric stabilisation due to the presence of adsorbed humic macromolecules (Saleh et al., 2008)and was consistent with previous observations (Buffle et al., 1998; Chen and Elimelech, 2007; Heidmann et al., 2004; Tipping and Ohnstad, 1984). Humic and fulvic acid molecules have been reported to associate with fullerenes and MWCNTs through π-π interactions between the cross-linked aromatic networks (Hyung et al., 2007; Hyung and Kim, 2008; Terashima and Nagao, 2007). Similarly, Saleh et al. (2010) investigated the effect of biomacromolecules and humic acid on the aggregation kinetics of SWCNTs in the presence of electrolytes. The biomacromolecules used were sodium alginate, bovine serum albumin (BSA), a microbial culture medium [Luria-Bertani (LB) broth], and SRHA, in the presence of NaCl and $CaCl_2$ salts. SWCNT stability curves were constructed using the aggregation kinetics data from which the critical coagulation concentrations for the monovalent and divalent salts were determined. The SWCNT preparation protocol and the aggregation studies were conducted in the same manner as the MWCNTs (Saleh et al., 2008). It was demonstrated that the mechanism of stabilisation of the SWCNTs in aqueous media followed DLVO type interactions. The *ccc* values reported were 20 mM NaCl and ~2 mM $CaCl_2$, which were lower than reported values in the literature for SWCNTs and MWCNTs (Saleh et al., 2008; Sano et al., 2001). The authors observed reduction in the aggregation rate of SWCNTs in the presence of biomacromolecules and SRHA, in the order; BSA > SRHA > LB > alginate. It was further observed that for the salt concentrations examined, none of the stability profiles with biomacromolecules or SRHA reached the favourable aggregation regime, demonstrating that the presence of biomacromolecules and SRHA enhanced the stability of SWCNTs even at high salt concentrations. The authors observed at high $CaCl_2$ concentrations, the aggregation of SWCNTs increased in the presence of alginate resulting in attachment efficiencies greater than 1. This was attributed to alginate molecule bridging of SWCNTs with Ca^{2+}.

Smith et al. (2009) conducted a similar study with oxidised MWCNTs prepared by refluxing pristine MWCNTs in nitric acid, reporting a similar influence of counterion concentration and valence on the observed aggregation behaviour. Their work differed from that of Saleh et al. (2008) only in the oxidation of the MWCNTs. The influence of natural organic matter was also not investigated. The *ccc* values obtained using TRDLS in the presence of Na^+, Mg^{2+} and Ca^{2+} were 93 ± 5 mM, 1.8 mM and 1.2 mM, respectively. While these values were similar to those reported by Saleh et al. (2008) in the case of the divalent salts, the monovalent NaCl gave a much higher *ccc* value. Peng et al. used UV-visible absorption to study the stability of nitric acid oxidised MWCNTs over a 30 day period and investigated the influence of salts. Their investigations showed that during the 30 day aging time, the oxidised MWCNTs dispersion showed characteristic UV-vis absorbance peaks at 252 nm with the MWCNTs concentration reducing to 85% of the initial concentration after 30 days (Peng et al., 2009). An apparent dependence on counterion valence was observed in agreement with the Schulze-Hardy rule. The minimum concentration of Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe^{3+} and Al^{3+} required to cause precipitation in the MWCNT dispersions were determined based on a previously published procedure and termed precipitation value (Peng et al., 2009). This value is similar to *ccc* values, however their determination may not be as accurate as the procedure for *ccc* determination. Oxidised CNT dispersions were mixed with different concentrations of salt solution at 6.0 ± 0.2 after 2 hours of standing. The minimum salt concentration at which oxidised CNTs precipitated was determined as the precipitation value (Peng et al., 2009). The reported values for Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe^{3+} and Al^{3+} were 185 mM, 125 mM, 1.05 mM, 0.55 mM, 0.15 mM, and 0.036 mM respectively.

Addo Ntim et al. investigated the colloidal behaviour of aqueous dispersions of carboxylated MWCNT (MWCNT-COOH) in comparison with PVP wrapped MWCNTs (MWCNT-PVP). An n-octanol/water partitioning was used to establish a hydrophobicity index (HI) for the two MWCNT derivatives. The HI was calculated using the light absorbance of the CNT dispersions at a wavelength of 252 nm in deionised water and in the presence of electrolytes before and after extraction (Addo Ntim et al., 2011). It was observed that MWCNT-PVP was more hydrophilic in DI water than the MWCNT-COOHs [Figure 4(a)] and the presence of salt results in significantly increased HI with MWCNT-COOH recording higher values than MWCNT-PVP [Figure 4(a)].

Figure 4 (a) HI of the MWCNT derivatives in deionised water, NaCl, NaOAc and $MgCl_2$ (b) Particle size distribution as a function NaCl concentration (see online version for colours)



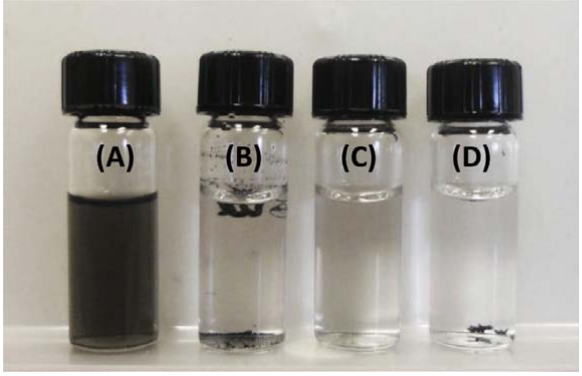
(a) (b)

Source: Addo Ntim et al. (2011)

A TRDLS method was used to compare the aggregation behaviour of the above mentioned MWCNT derivatives in the presence of Na^+ and Mg^{2+} (Addo Ntim et al., 2011). A dependence on surface modification on the aggregation kinetics of functionalised MWCNTs was established. The polymer wrapped system was generally less sensitive to ionic strength than the carboxylated analogues where a rapid increase in hydrodynamic size with increasing ionic strength was not observed [Figure 4(b)]. The water dispersibility of the MWCNT-COOHs originates from the negatively charged oxygen-containing groups on the CNT surface whereas the CNTs in the MWCNT-PVP dispersion were sterically stabilised by the presence of the hydrophilic polymer on the surface. This accounted for the difference in the aggregation behaviour. MWCNT-COOH showed higher attachment efficiencies than MWCNT-PVP and the *ccc* values obtained were 0.6 mM and 18 mM Mg^{2+} for MWCNT-COOH and MWCNT-PVP respectively, and the corresponding values in NaCl were 125 mM and 100 mM (Addo Ntim et al., 2011).

Desai et al. demonstrated that organic soluble, hydrophobic, functionalised MWCNTs could potentially be water pollutants through an antisolvent precipitation process. Dispersions of ODA functionalised MWCNTs prepared by antisolvent precipitation in ethanol, acetonitrile (ACN), tetrahydrofuran (THF) and acetone where water served as the antisolvent were observed to be fairly stable over a period of 24 hours (Figure 5) (Desai et al., 2012). TRDLS was used to study the aggregation behaviour of the dispersions in the presence of NaCl and it was observed that attachment efficiency increased linearly with salt concentration at low salt concentrations without really reaching a steady state, which was not in agreement with the DLVO theory. Pseudo-*ccc* values were therefore proposed for the water/solvent systems at the point where the kinetic curve changed slope. The pseudo-*ccc* values reported were 90 mM, 55 mM, 60 mM, and 30 mM for THF/water, ethanol/water, acetone/water and ACN/water respectively. These were significantly lower than *ccc* values observed for hydrophilic carboxylated and polymer wrapped MWCNT previously reported (Addo Ntim et al., 2011; Peng et al., 2009; Smith et al., 2009).

Figure 5 (a) MWNT-ODA dispersed in THF (10 ppm) (b) Precipitation of MWNT-ODA in water (c) Dispersed MWCNT-ODA after antisolvent precipitation (d) Partial precipitation of dispersed MWCNT-ODA after addition of NaCl (see online version for colours)



Source: Desai et al. (2012)

While the *ccc* values in the presence of divalent and trivalent salts appeared to be reproducible across the studies reviewed, the values obtained for monovalent salts varied quite a bit (Addo Ntim et al., 2011; Saleh et al., 2008, 2010; Sano et al., 2001; Smith et al., 2009). Generally, values obtained for as-prepared CNTs were lower than oxidised ones.

The effect of size on colloidal stability of CNTs is an important issue that is yet to be studied in sufficient details. This is especially true because several cytotoxicological studies have reported an effect of size on the toxicity of nanoparticles (Karlsson et al., 2009; Napierska et al., 2009; Pan et al., 2007; Yuan et al., 2010). Most of these studies observed more toxic effects in smaller size ranges than the larger ones. It is important to realise that CNTs represent a diverse group of tubular structures that vary in size, shape and chirality (Figure 1). For example, in recent years there has been interest in shorter CNTs for high field quasiballistic electron transport (Javey et al., 2004), hydrogen storage (Liu et al., 2003), and energy storage systems such as in lithium batteries (Wang et al., 2007). Since size alters many of the properties of CNTs, it may also affect its fate and transport and is an important parameter when CNTs are in consideration as pollutants.

Heister and co-workers demonstrated that the dimensions of the CNTs after oxidation clearly influence the stability of the resulting CNT dispersions. In a study of the stability of different CNT samples sorted by length according to AFM and PALS data the following groups: oxidised SWCNT from Nanolab (oxSWCNTs Nanolab), double walled CNTs (oxDWNTs), MWCNTs [oxMWNTs (long and thin) and oxMWNTs (short and thick)] and SWCNT from CoMoCAT (oxSWCNTs CoMoCAT). It was demonstrated that oxSWCNTs Nanolab was the most sensitive to changes in buffer pH and concentration. Co-MoCAT, oxSWCNTs and short and thick MWNTs were the least sensitive to those parameters (Heister et al., 2010). This behaviour was attributed to increased intertube attraction within the longer tubes, increasing their propensity towards agglomeration and bundling in comparison to the short CNTs. To demonstrate the effect of aspect ratio on the genotoxicity of MWCNTs, Kim et al. used low-aspect-ratio MWCNTs and high-aspect-ratio MWCNTs in bacterial reverse mutation, *in vitro* chromosome aberration, and *in vivo* micronuclei tests. It was observed that low-aspect-ratio MWCNTs were generally better dispersed than the high-aspect-ratio MWCNTs in distilled water and dipalmitoylphosphatidylcholine (DPPC). However neither the high-nor the low-aspect-ratio MWCNTs were observed to induce any genotoxicity in both *in vitro* and *in vivo* tests, although the high-aspect-ratio MWCNTs were reportedly more toxic than the low-aspect-ratio MWCNTs (Kim et al., 2011). These studies demonstrated an influence of the dimensions of CNTs on their dispersibility which may translate into influencing their toxic effects. More studies into the size dependent toxicity of CNTs are therefore necessary.

In summary, data published on the aggregation behaviour of CNTs suggest that they can be relatively stable in the solution chemistries typical of aquatic environments, and the presence of natural organic matter and other macromolecules may enhance their aqueous dispersibility and therefore their persistence in aquatic environments.

4 Dispersibility in biological media and influence on toxicological effects

4.1 Aggregation behaviour in biological media

Physiological fluids contain various proteins and other organic molecules, as well as high salt concentrations, and sometimes variation in pH, making it extremely important for CNT dispersions intended for *in vitro* or *in vivo* experiments to be stable under the prevailing conditions. The review of the aggregation behaviour of CNTs in the presence of electrolytes has established that the solubility of CNTs depends on the pH, decreases with increasing salt concentration, and the presence of biomacromolecules may influence their aggregation behaviour. Some of the key factors involved in CNT aggregation are hydrophobicity, attractive van der Waals forces, and the potential influence of electrical double layer formation on the surface of functionalised CNTs (Girifalco et al., 2000). The hydrophobic surface properties of CNTs make them associate readily with biological macromolecules, such as proteins (Karajanagi et al., 2004; Shi Kam et al., 2004; Shim et al., 2002) and antibodies (Sager et al., 2007), as well as natural organic matter, such as humic substances (Hyung et al., 2007). The interaction of CNTs with macromolecules is inevitable in biological systems including culture media that contain macromolecules such as enzymes, proteins, DNA, and polysaccharides. Dispersing agents have been used in the preparation of relatively well dispersed CNT suspensions. A number of investigators have studied the aggregation behaviour of CNTs in culture media with particular emphasis on the effect of macromolecules, polymers and surfactants.

In an effort to develop quantitative methods for assessing CNT dispersibility in water and tissue culture media, Wang et al. (2010) investigated the dispersibility of three MWCNTs derivatives: as-prepared (AP-MWCNT), purified (PD-MWCNT) and carboxylated (COOH-MWCNT). It was that demonstrated partitioning coefficient, suspension stability index, attachment efficiency, and dynamic light scattering (DLS) could be used systematically in preparing stable MWCNT suspensions through the use of the protein bovine serum albumen (BSA) and the phospholipid DPPC. The HI for the different MWCNT derivatives was calculated using the light absorbance of the CNT dispersions in distilled H_2O, phenol red-free Dulbecco's modified eagle's medium (DMEM) and bronchial epithelial basal medium (BEBM) before and after n-octanol extraction. It was demonstrated that AP-MWCNTs and PD-MWCNTs had relatively higher hydrophobicity indices than the COOH-MWCNTs, which was in agreement with their zeta potential values, where COOH-MWCNT had a relatively higher negative value compared to AP-MWCNT and PD-MWCNT. This was attributed to electrostatic repulsion by surface carboxyl groups present in COOH-MWCNT which was non-existent in others. Following the addition of BSA, the zeta potential values of the aqueous dispersions of AP-MWCNT and PD-MWCNT were observed to significantly increase, which was attributed to direct binding of BSA to the hydrophobic tube surfaces providing steric stabilisation (Chen et al., 2003) and also electrosteric hindrance as a result of the anionic nature of BSA at neutral pH. The influence of BSA on the stability of the more hydrophilic COOH-MWCNT was however attributed to steric hindrance. The aggregation kinetics of COOH-MWCNT in PBS was investigated with and without BSA, and it was observed that the presence of BSA resulted in significantly lower attachment efficiencies indicating a significant contribution of BSA towards stabilising COOH-MWCNT. This was attributed to a combination of steric and electrosteric hindrances from attached BSA and possible Ca^{2+} bridging of the protein to the CNT surface. UV-visible spectroscopy was used to establish a stability index for the CNTs in bronchial epithelial growth medium (BEGM) and DMEM and after addition of BSA and DPPC to assess their influence on the stability of the MWCNT derivatives (Wang et al., 2010). It was observed that while BSA significantly improved the stability of the CNTs, DPPC showed no improvement. A combination of BSA and DPPC was observed to synergistically improve the stability of the more hydrophobic CNTs.

Kim et al. investigated the macrodispersion of SWCNTs and MWCNTs in the presence of BSA, tween 80, DPPC, and dimethylsulfoxide (DMSO) using a light absorbance method (Kim et al., 2011). The dispersion stability of the dispersed CNTs evaluated over a period of 16 weeks indicated that BSA was the best among the dispersants studied for both SWCNT and MWCNT which was in agreement with the data reported by Wang et al. (2010). Heister et al. demonstrated an influence of CNT dimensions and surface functionalities on their aqueous dispersibility and in vitro behaviour. They investigated the effect of CNT dimensions and surface properties on their dispersion stability in salt solutions at different pHs and concentrations and in biological fluids, such as cellular growth media and human plasma, and their toxicity toward cancer cells. They also demonstrated that oxidation debris arising from the functionalisation process tends to enhance stability of CNTs in dispersions by acting as a pseudo-surfactant or charge buffer (Heister et al., 2010).

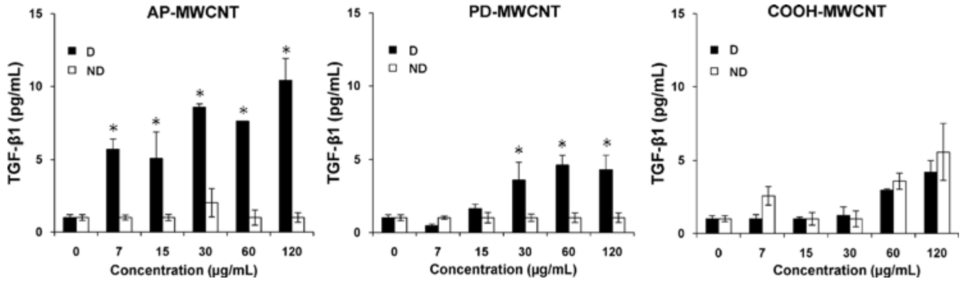
4.2 Influence of dispersal state on toxicological effects

One of the most prevalent challenges to evaluating in vitro and in vivo toxicity of CNTs as has been discussed above is dispersion since the aggregation or agglomeration of CNTs in culture media makes it difficult to effectively interpret the results obtained from toxicity studies. As a result several studies have looked at the conditions under which CNT dispersibility can be preserved in physiological media during such toxicity analysis. Several studies have successfully incorporated various dispersing agents and have compared the effect of dispersal state of CNTs on their toxicity to cells. As discussed earlier the components of cell culture media such as proteins and phospholipids have been reported to affect the dispersal state of CNTs (Wang et al., 2010). The effect of serum components (amino acids, vitamins, phenol red and FBS) on the dispersal state of SWCNTs, their debundling and subsequent toxicity to A549 cells have been reported (Casey et al., 2007). From ramanspectroscopy data, it was demonstrated that although components of the media improved the dispersal state of the SWCNTs, they were unable to cause any significant debundling of the SWCNTs. Muller et al. demonstrated that better dispersed MWCNTs obtained by grinding were more biopersistent in the lung than intact MWCNTs and therefore had greater potential to induce inflammatory and fibrotic reactions (Muller et al., 2005).

Wang et al. (2010) demonstrated that BSA and DPPC function in an additive fashion to disperse hydrophobic AP-MWCNTs in an epithelial growth medium leading to increased transforming growth factor β1 (TGF-β1) production in a bronchial epithelial cell line. Better-dispersed MWCNTs were also observed to increase fibroblast proliferation, consistent with previous observations in the rodent lung (Meng et al., 2009; Shvedova et al., 2005). The effects of the dispersion state of MWCNTs on cellular behaviour, their influence on cell viability, LDH release and the production of

TGF-β1 in bronchial epithelial cell line (BEAS-2B) was investigated. It was observed that although neither agglomerated nor dispersed tubes had any effect on cellular metabolic activity or lactate dehydrogenase (LDH) release, TGF-β1 production increased in response to well dispersed AP-MWCNTs and PDMWCNTs, which was not observed in agglomerated tubes that were not treated with a dispersant (Figure 6). The addition of dispersants to COOH-MWCNTs had no effect on TGF-β1 production (Wang et al., 2010).

Figure 6 TGF-β1 release by BEAS-2B cells compared between dispersed (D) and non-dispersed (ND) MWCNTs



Note: The asterisks (*) denote $p < 0.05$, comparing non-dispersed (ND) to dispersed (D) tubes.

Source: Wang et al. (2010)

The effect of some commonly used surfactants on dispersal state-related cytotoxicity have been reported (Monteiro-Riviere et al., 2005). The toxic effects of MWCNTs dispersed with pluronic L61, pluronic L92, pluronic F127, tween 20, and tween 60 on HEK keratinocytes were compared. Due to the intrinsic toxicity exhibited by the other four surfactants in preliminary analysis only pluronic F127 was used in further toxicity analysis. It was observed that cell viability was reduced to a comparable extent in the presence of MWCNTs dispersed with or without the surfactant. It was however observed that interleukin 8 (IL-8) production was reduced in the presence of the surfactant dispersed MWCNTs although independently both MWCNTs and the surfactant caused an increase in IL-8 production. The authors suggested that a more complex interaction between the MWCNTs and the surfactant than mere dispersion may be responsible for the observed outcomes (Monteiro-Riviere et al., 2005).

In an *in vitro* cytotoxicity study with human MSTO-211H cells, Wick et al. demonstrated that SWCNTs at various degrees of agglomeration induced different levels of toxicity. Polyoxyethylene sorbitan monooleate (PS80) dispersed SWCNT bundles were reported to induce less cytotoxic effects than asbestos fibres, whereas as-prepared SWCNT and conventionally purified rope-like agglomerates were more toxic (Wick et al., 2007). The comparative toxic effects of SWCNTs dispersed with and without acetone treatment on the lungs have been reported (Mercer et al., 2008). Aggregates of untreated SWCNTs were observed in lung micrographs of exposed mice, encased by macrophages to form granulomatous inflammation. Lung micrographs of acetone dispersed SWCNTs were however devoid of such inflammations (Mercer et al., 2008). A similar observation was made by Wang et al. (2011), who demonstrated that MWCNT dispersal state influenced profibrogenic epithelial and macrophages responses that correlate with the extent and localisation of pulmonary fibrosis. In an *in vitro* and *in vivo* analysis of three different MWCNT derivatives (AP-MWCNT, PD-MWCNT and COOH-MWCNT), tube uptake by BEAS-2B cells, THP-1 cells and alveolar macrophages, it was observed that the CNTs that were well-dispersed with BSA and DPPC induced TGF-β1 and platelet-derived growth factor AA (PDGF-AA) production in the lung in parallel with prominent granulomatous inflammation and fibrosis around small airways, which was not as pronounced in the case of their non-dispersed counterparts (Wang et al., 2011). These studies suggest a relationship between the dispersal state of CNTs and their observed toxic effects. It is probably that aggregation affects the effective dose of CNTs in toxicity studies such that the actual amount of CNTs available to cause toxicity in the cells is reduced, resulting in reduced toxicity. However some studies have also shown the opposite effect where better dispersed CNTs have shown little or no toxic effects, as demonstrated by Wang et al. (2011), where the highly hydrophilic COOH-MWCNT were poorly taken up by BEAS-2B cells and induced little or no TGF-β1 production in vitro studies. While the hypothesis of an improved dispersal state allowing CNTs better access to cells may be plausible, the relationship between the state of CNT dispersal, their bioavailability and therefore the mechanism by which toxicity is induced is still not very clear.

5 Conclusions

Developing an understanding of the aggregation behaviour of CNTs in aquatic and biological environments is essential to providing insight into the fate, transport and risk associated with these nanomaterials. It should be noted that CNTs represent a highly non-ideal system, where the individual particles are cylindrical and they form aggregates by entangling with each other. Results from dispersion studies point towards a DLVO-type aggregation behaviour typical of most colloidal systems. This is especially true for oxidised CNTs where aggregation rate increases with increasing electrolyte concentration until the diffusing-limited region where aggregation is no longer dependent on salt concentration. The dispersal state of CNTs is also one of the most important physicochemical characteristics that need attention during cytotoxicological studies because the interactions at the nano-bio interface depend upon it. The relationship between dispersal state and toxicological effects are just beginning to emerge. While significant effort has gone into these studies, much is unknown as far as colloidal stability as a function CNT size, morphology and functionalisation.

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