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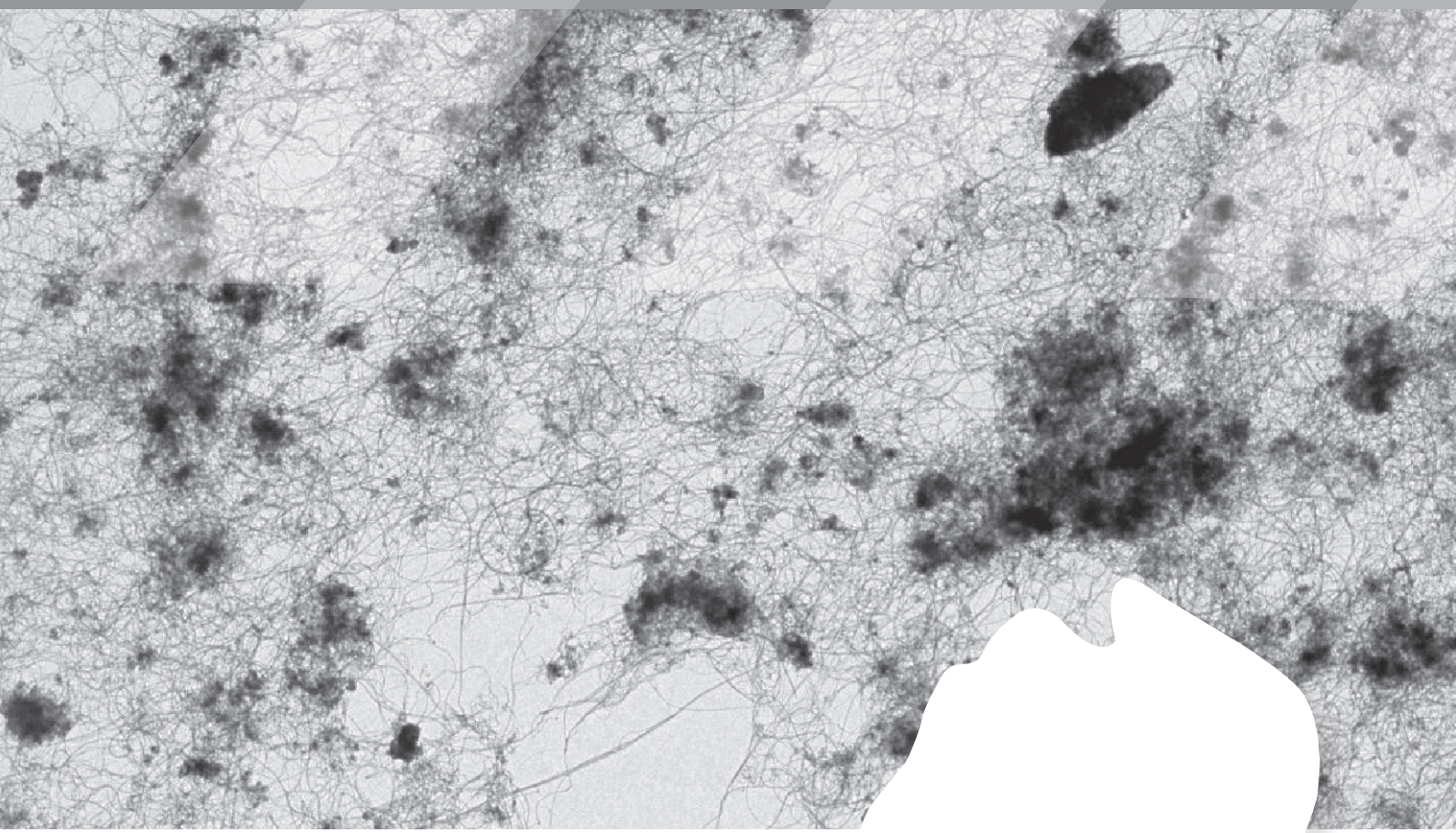
# Durability of carbon nanotubes and their potential to cause inflammation



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## Executive Summary

It has been suggested that carbon nanotubes (CNTs) might conform to the fibre pathogenicity paradigm that explains the pathogenicity of asbestos and other fibres on a continuum based on length, aspect ratio and biopersistence. Multiwalled carbon nanotubes have shown that they may satisfy the first two aspects of the fibre paradigm but biopersistence has not been investigated to a substantial degree for carbon nanotubes in general. Whilst biopersistence is complex and requires animal studies, durability, the chemical mimicking of the process of fibre dissolution using *in vitro* treatment, is a rapid alternative that is easier to determine and is closely related to biopersistence but does not require large numbers of animals.

The University of Edinburgh, the Institute of Occupational Medicine (Edinburgh), and the Commonwealth Scientific & Industrial Research Organisation (Australia) have collaborated to determine the durability of carbon nanotubes in simulated biological fluid and subsequent fibre pathogenicity, compared with well-characterised fibre controls. This collaborative project was financially supported under Safe Work Australia's Nanotechnology Work Health and Safety Program.

The study had two main components. First, known masses of four types of carbon nanotubes (one type of single-walled CNT ($CNT_{SW}$) and three types of multi-walled CNTs ($CNT_{SPIN}$, $CNT_{TANG2}$ and $CNT_{LONG1}$)), two types of asbestos fibres (one type of chrysotile asbestos (LFC) and one type of amosite asbestos (LFA)), and one type of glass wool fibre (X607) were incubated in simulated biological fluid (Gambles solution) for up to 24 weeks, with samples removed from incubation, filtered, dried, and weighed at defined time-points (0wk, 3wk, 6wk, 10wk and 24wk). Two of the four types of carbon nanotubes, $CNT_{SPIN}$ and $CNT_{SW}$, showed no loss of mass, and no change in morphology or average fibre length when viewed by electron microscopy. A third, $CNT_{TANG2}$, showed a possible loss of mass (~25%) after 24wk incubation, but no change in morphology. However, the fourth type of carbon nanotube, $CNT_{LONG1}$, was recovered at only 70% of original mass at all time-points from 3wk onwards. Electron microscopy confirmed that the average fibre length had decreased slightly and the proportion of long fibres had also decreased, indicating that this type of nanotube had undergone some kind of modification during the incubation in Gambles solution that had resulted in fibre dissolution and/or breakage.

The second part of the study was designed to investigate the impact of incubation in Gambles solution on carbon nanotube pathogenicity in mice in comparison to the asbestos and glass wool fibres. Two types of CNT samples included in the durability assessment ($CNT_{SPIN}$ and $CNT_{TANG2}$) were excluded from *in vivo* assessment leaving one type of multi-walled CNT ($CNT_{LONG1}$) and one type of single-walled CNT ($CNT_{SW}$). The $CNT_{LONG1}$ sample had previously been shown to induce an asbestos-like response when injected into the peritoneal cavity of mice, a model for the biological response of exposure at the mesothelium following inhalation of high aspect ratio fibres. The incubation in Gambles solution and subsequent loss of mass and fibre shortening for $CNT_{LONG1}$ was associated with decreased pathogenicity compared to the strong inflammatory and granuloma response induced in mice by injection of $CNT_{LONG1}$ fibres that had been incubated for 0wk. A similar loss of pathogenicity with incubation was also observed for the less durable of the asbestos fibres included here (LFC), whereas the second, more durable asbestos (LFA) showed no significant loss of mass, no change in morphology and no loss of pathogenicity. The $CNT_{SW}$ sample was shown by electron microscopy to form very tightly agglomerated particle-like bundles and did not elicit an inflammatory response in mice regardless of incubation.

These results, therefore, support the view that carbon nanotubes can be durable but may also be subject to bio-modification in a sample-specific manner. They also suggest that if they are of sufficient length and aspect ratio, pristine carbon nanotubes can induce asbestos-like responses in mice, but that this may be mitigated if the nanotubes are of a less durable nature.

## Glossary

| | |
|---|---|
| ANOVA | analysis of variance |
| BCA | bicinchoninic acid |
| BSA | bovine serum albumin |
| CNT(s) | carbon nanotube(s) |
| d | day(s) |
| $ddH_2O$ | double-distilled water |
| EDXA | energy-dispersive x-ray analysis |
| ELISA | enzyme-linked immunosorbent assay |
| EPR | electron paramagnetic resonance |
| g | gram |
| h | hour(s) |
| HPR | horseradish peroxidase |
| ICP-AES | inductively-coupled plasma atomic emission spectroscopy |
| ICP-MS | inductively-coupled plasma mass spectrometry |
| IL-6 | interleukin-6 |
| L | litre |
| LAL | limulus amebocyte lysate |
| LDH | lactate dehydrogenase |
| LFA | long fibre amosite |
| LFC | long fibre chrysotile |
| MAC | macrophages |
| mg | milligram |
| min | minute(s) |
| ml | millilitre |
| mm | millimetre |
| mM | millimolar |
| MWCNT(s) | multi-walled carbon nanotube(s) |
| nm | nanometre |
| pg | picogram |
| PMN | polymorphonuclear leukocytes |
| rpm | revolutions per minute |
| SEM | scanning electron microscopy |
| SE | standard error (of the mean) |
| sec | second(s) |
| SWCNT(s) | single-walled carbon nanotube(s) |
| TEM | transmission electron microscopy |
| v/v | volume/volume |
| wk | week |
| µl | microlitre |
| µm | micrometre |

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# 1. Introduction

Carbon nanotubes (CNTs) are a new form of commercially exploited nanoparticle and can essentially be described as seamless cylinders of rolled up graphite. CNTs occur in two classes: single-walled CNTs (SWCNTs), where each CNT is composed of one cylinder, and multi-walled CNTs (MWCNTs), where each CNT comprises multiple cylinders stacked concentrically inside one another. SWCNTs, with diameters generally ~1-5 nm, are typically narrower than MWCNTS, which have diameters more typically ranging between 5-100nm. Lengths of either type can reach into the micron range [1]. The properties of engineered CNTs, including exceptional strength, lightness and conductivity, may make them valuable for their potential industrial and medical applications [2, 3]. However their structural similarities with asbestos were quickly noticed, raising concerns about possible health effects on humans [4].

Of particular importance to the toxicology of CNTs is their potential to be manufactured to pathogenic lengths with high aspect ratios (high length: width ratio) and resistance to chemical degradation. It has thus been suggested that the potential pathogenicity of CNTs might conform to the 'fibre pathogenicity paradigm', by which the pathogenicity of a fibre can be predicted on a continuum based on its length, aspect ratio and biopersistence [5]. On this continuum, fibres that are more likely to induce 'asbestos-like' pathologies such as asbestosis, lung cancer, and mesothelioma will be narrow enough that they can reach the distal lung upon inhalation, long enough to be incompletely engulfed by macrophages during clearance, and resistant to chemical attack or breakage. Deposition of these types of biopersistent fibres in the distal lung region is problematic because clearance here is predominantly mediated by macrophages, which, if they are unable to successfully clear the long fibres, will enter a state of chronic stimulation (frustrated phagocytosis) with concomitant release of inflammatory mediators, leading to the development of fibrosis and possibly other pathologies [1, 6]. Fibres >20μm long are generally regarded on the continuum as being of sufficient length to induce frustrated phagocytosis whereas fibres <5μm can be cleared from the body as particles [7]. CNTs can easily be manufactured to reach these lengths or longer and, although they are generally prone to form sticky aggregates due to van der Waals forces, if individual fibres are aerosolised, their physical structure might allow them to align lengthwise with the airstream and thus reach the distal lung [5].

Biopersistent fibres resist the leaching out, or solubilisation of, structural elements within a biological environment such as the lung lining fluid or the internal environment of macrophages. Less persistent fibres, in contrast, can weaken and break, and thus become short enough for complete phagocytosis and clearance [6]. Biopersistence is now regarded as one of the most important determinants of fibre pathogenicity [5] (Figure 1).

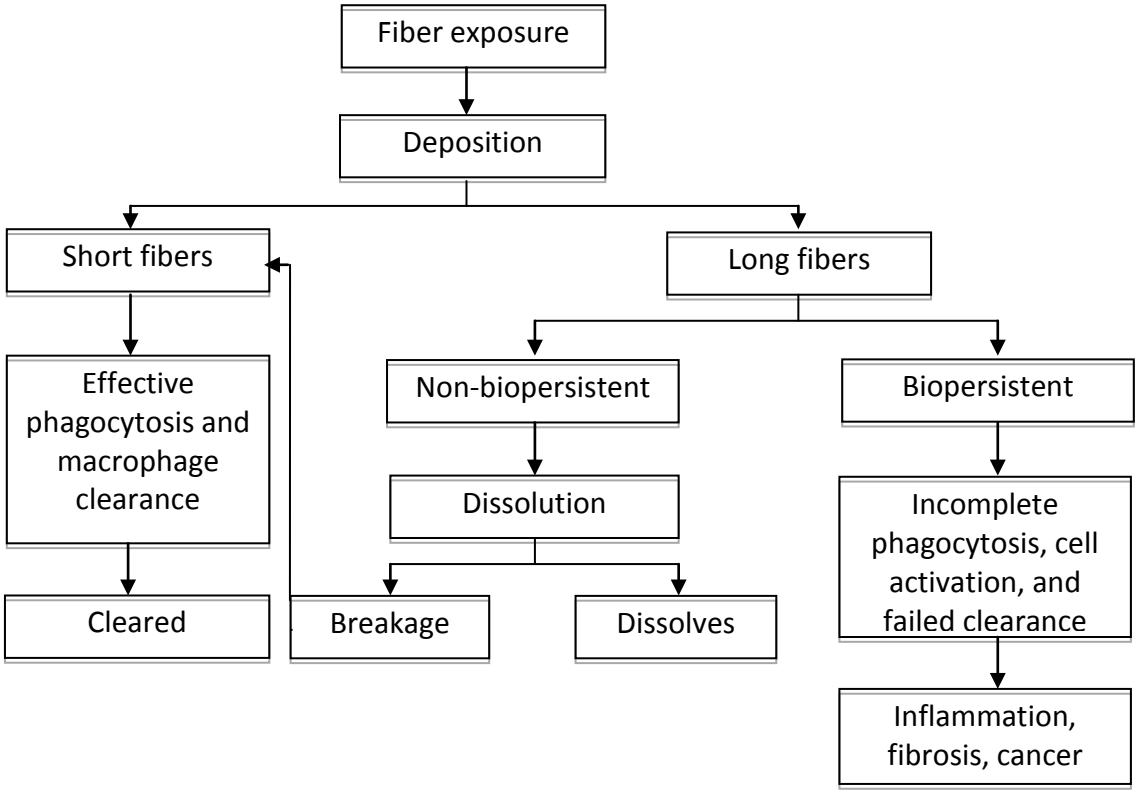


**Figure 1. Paradigm for the role of long fibres and biopersistence in the pathogenic effects of fibres (from Donaldson *et al.* 2006)**

One approach by which a fibre's biopersistence can be inferred is to measure its durability, whereby samples of the fibre are assessed for loss of mass over time in a simulated biological environment. Leaching or solubilisation of structural elements associated with fibre breakdown or dissolution can be indirectly determined by loss of mass and this approach has been used with success to predict biopersistence [8].

This report describes and discusses experiments designed to assess the durability of four types of CNTs compared to two types of asbestos fibres and one type of glass wool fibre, and the inflammogenicity of a subset of these. These experiments were conducted in collaboration by the University of Edinburgh, the Institute of Occupational Medicine (Edinburgh), and the Commonwealth Scientific & Industrial Research Organisation (CSIRO, Australia), commissioned by the Australian Government's Safe Work Australia as part of its Nanotechnology Work Health & Safety Program.

# 2. Materials and methods

## 2.1 Test samples used in this study

A panel of seven fibre or CNT types was used in this study (Table 1). This panel comprised four types of CNTs [CNT single-walled ($CNT_{SW}$); spinnable CNT multi-walled ($CNT_{SPIN}$); "long" CNT multi-walled ($CNT_{LONG1}$); "tangled" CNT multi-walled ($CNT_{TANG2}$)], as well as two types of asbestos [long fibre amosite (LFA); long fibre chrysotile (LFC)], and one type of long glass fibre (X607).

**Table 1. Test samples used in this study & rationale for use**

| Fibre | Rationale for Use |
|---|---|
| X607 | Non-durable long-fibre control |
| LFA | Durable amosite asbestos control |
| LFC | Non-durable chrysotile asbestos control |
| $CNT_{SW}$ | Unknown Durability |
| $CNT_{SPIN}$ | Unknown Durability |
| $CNT_{LONG1}$ | Unknown Durability |
| $CNT_{TANG2}$ | Unknown Durability |

### 2.1.1 Characteristics of test samples used in this study

Two of the test samples used in this study ($CNT_{LONG1}$ and $CNT_{TANG2}$) have been previously described in Poland *el al.* (2008). These two samples were characterised again here to maintain consistency between all samples used here.

Fibre and CNT size and shape were characterised by scanning and transmission electron microscopy (SEM and TEM, respectively) and chemical characteristics were confirmed by energy-dispersive x-ray analysis (EDXA).

For quantification of contaminating metals, approximately 0.01g/ml of each sample was weighed into an acid-cleaned digestion tube containing 0.2% (v/v) $HNO_3$. Tubes were mixed and incubated at room temperature for 15 min after which the 0.2% $HNO_3$ leach solution was filtered through acid-cleaned 0.45µm filter cartridges and analysed by matrix matched standards using the techniques of inductively-coupled plasma mass spectrometry (ICP-MS) or inductively-coupled plasma atomic emission spectroscopy (ICP-AES).

For measurement of endotoxin levels, 1mg/ml each sample was vortexed for 1 min in limulus amebocyte lysate (LAL) endotoxin-free water and incubated for 1h at 37˚C. Samples were then centrifuged and endotoxin levels in the supernatant were determined in triplicate using the QLC-1000 Chromogenic LAL kit (Lonza, Australia) following manufacturer's instructions. A trial had previously shown that centrifugation did not artificially lower endotoxin levels in the supernatant. An aliquot of each supernatant was also spiked with a known amount of endotoxin and measured alongside unspiked samples to confirm the absence of assay inhibition.

Samples were assessed for the potential to generate free radicals by electron paramagnetic resonance (EPR). TEMPONE-H (Enzo Life Sciences) was used as a spin trap to quantify peroxynitrite and superoxide radical formation. Samples were prepared by diluting the filtered test samples that had been resuspended for injection (2.3.4) to 0.01mg/ml in saline. TEMPONE-H (1μl of 100mM stock solution) was added to 99μl of the diluted test sample to obtain a final concentration of 1mM TEMPONE-H. The samples were incubated at 37° for 60 min after which the levels of oxidised TEMPONE-H were quantified by EPR. Undiluted test samples at a presumed mass of 0.5mg/ml were also assessed. Pyrogallal in Hanks Buffered Solution was a positive control for the TEMPONE-H reaction. A negative control for the 0.01mg/ml samples for the TEMPONE-H reaction was prepared by adding 2μl 0.05% bovine serum albumin (BSA):saline to 97μl saline. A negative control for the undiluted test samples was prepared by using 99μl 0.05% BSA:saline.Test sample characteristics are described as fully as possible in Table 2.

**Table 2. Physical and chemical characteristics of the test samples used in this study**

| | Morphology | Diameter as supplied by manufacturer (nm, mean±SE) | Diameter as determined by the authors (nm, mean±SE) | Length as supplied by manufacturer (μm) | Length as determined by the authors (μm, ±SE) | %Fibres greater than 15μm | %Fibres greater than 20μm | Endotoxin[1] (pg ml$^{-1}$) | Soluble metals (μg g$^{-1}$) | Free radical generation | Description of Morphology (SEM, TEM, Light Microscopy) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **X607** | Glass fibres | NA | 3500±2000 | NA | 123±69 | 98 | 94 | ND | Li-0.41; Be-0.02; Al-350[2]; V-1.07; Cr-4.05; Mn-29.6; Fe-314[2]; Co-0.08; Ni-2.62; Cu-0.72; Zn-7.09; As-0.12; Sr-48.4; Mo-0.25; Ag-<0.01; Cd-0.02; Sb-0.01; Pb-0.43; U-0.03 | None | Dispersed rod-like glass fibres. |
| **LFA** | Amphibole asbestos | NA | 550±390 | NA | 34±36 | 63 | 54 | ND | Li-0.15; Be-0.15; Al-463; V-1.62; Cr-4.45; Mn-622; Fe-~3200[3]; Co-0.45; Ni-2.67; Cu-2.96; Zn-2.9; As-0.07; Sr-29.2; Mo-0.45; Ag-<0.03; Cd-0.02; Sb-<0.04; Pb-0.79; U-0.04 | None | Dispersed rod-like amphibole asbestos. |
| **LFC** | Chrysotile asbestos | NA | 42±12 | NA | 11±12 | 26 | 20 | ND | Li-<0.14; Be-0.01; Al-137; V-1.29; Cr-32.3; Mn-83.1; Fe-1220; Co-5.54; Ni-140; Cu-0.77; Zn-10.9; As-<0.11; Sr-2.26; Mo-0.08; Ag-<0.08; Cd-0.04; Sb-<0.09; Pb-0.46; U-0.02 | None | Dispersed fibrous-looking chrysotile asbestos. |
| **$CNT_{SPIN}$** | Multi-walled | 8-10 | 9±3 | 200-300 | NAs[4] | NAs | NAs | ND | Li-<0.02; Be-<0.001; Al-0.3; V-0.01; Cr-0.07; Mn-0.02; Fe-50.1; Co-<0.003; Ni-0.46; Cu-0.16; Zn-0.95; As-0.12; Sr-48.4; Mo-0.25; Ag-<0.01; Cd-0.02; Sb-0.01; Pb-0.43; U-0.001 | None | Agglomerated sheets of very long fibres with a hair-like appearance. |
| | **Morphology** | **Diameter as supplied by manufacturer (nm,** | **Diameter as determined by the authors (nm,** | **Length as supplied by manufacturer** | **Length as determined by the authors (μm,** | **%Fibres greater than 15μm** | **%Fibres greater than 20μm** | **Endotoxin[1] (pg ml$^{-1}$)** | **Soluble metals (μg g$^{-1}$)** | **Free radical generation** | **Description of Morphology (SEM, TEM, Light Microscopy)** |

| | | mean±SE) | mean±SE) | (μm) | ±SE) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **$CNT_{SW}$** | Single-walled | 1-2 | 5±2 | 0.5-2 | 4±2 | 0 | 0 | ND | Li-<0.04; Be-<0.003; Al-6.2; V-0.34; Cr-2.01; Mn-15.7; Fe-185; Co-442; Ni-47.4; Cu-1.13; Zn-2.9; As-0.23; Sr-2.72; Mo-144; Ag-0.03; Cd-0.1; Sb-<0.03; Pb-0.98; U-0.04 | None | Bundles of tightly agglomerated SWNTs in which the presence of individual NTs could not readily be determined. |
| **$CNT_{LONG1}$** | Multi-walled | 40-50 | 64±16 | Mean 13 | 12±6 | 30. | 10 | ND | Li-<0.09; Be-<0.007; Al-0.9; V-0.01; Cr-0.15; Mn-0.09; Fe-15.6; Co-<0.01; Ni-0.2; Cu-0.06; Zn-2.5; As-<0.07; Sr-0.84; Mo-0.01; Ag-<0.05; Cd-<0.02; Sb-<0.06; Pb-0.03; U-0.01 | None | Dispersed bundles and singlets of long and intermediate-length MWNTs, many in the range 10-20μm and longer. Many very short fibres often decorate the long fibres. |
| **$CNT_{TANG2}$** | Multi-walled | 15 ± 5 | 10.3±5 | 5-20 | NAs | NAs | NAs | ND | Li-<0.08; Be-<0.006; Al-41.6; V-<0.01; Cr-0.03; Mn-0.05; Fe-606; Co-0.04; Ni-0.44; Cu-1.07; Zn-9.5; As-<0.07; Sr-0.3; Mo-655; Ag-<0.05; Cd-0.04; Sb-<0.06; Pb-0.26; U-<0.006 | None | Bundles of intermediate-length MWNTs. Often stellate in form with longer fibres protruding from the central tangled agglomerate, a large proportion of which are in respirable size range <5μm. |

ND = not detected
NA = not available
[1] Endotoxin detection limit <10 pg $ml^{-1}$
[2] Analysed by ICP-AES
[3] Value over-range and is estimate only
[4] NAs = Not assessable as ends of individual fibres could not be detected

(Expanded from Poland *et al*. 2008)

## *2.2 In vitro* assessment of durability

Gambles solution is a balanced electrolyte solution similar to the electrolyte environment of biological systems that is adjusted to mimic the pH inside macrophage phagolysosomes, the most potentially degradative pH that a particle should encounter following lung deposition and macrophage uptake. Gambles solution was prepared the day before addition to the samples (per litre: 7.12g NaCl; 1.95g $NaHCO_3$; 0.029g $CaCl_2.2H_2O$; 0.148g $Na_2HPO_4$; 0.079g $Na_2SO_4$; 0.212g $MgCl_2.6H_2O$; 0.118g Glycine; 0.152g $Na_3$-citrate.$2H_2O$; 0.18g $Na_2$-tartrate.$2H_2O$; 0.172g Na-pyruvate; 167μl lactic acid). Formaldehyde (2ml/L; 37% in formalin) was added to prevent microbial growth. The pH was adjusted by HCl to 4.5 then readjusted the next day prior to addition to the samples.

For each time-point of interest, four replicates of each test sample (each weighing 1.00-3.00mg) were weighed (OHaus AP2500) into 7ml plastic flat-bottomed Bijou tubes. An appropriate amount of Gambles solution was added to each sample to give a final concentration of 0.5mg sample/ml. Samples were sonicated (Fisherbrand ultrasonicating water bath, ultrasonic frequency: 40kHz) until the CNTs or fibres were visually judged to have dispersed as well as possible. Consequently, the X607, LFA and LFC samples were sonicated for 20 min and the CNT samples were sonicated for 1h. All samples were then incubated with shaking for up to 24 wk at 37°C with the Gambles solution refreshed every 3 wk. 0wk samples were treated identically to incubated samples with the exception that after mixing with Gambles solution they were immediately filtered and dried, without sonication.

The four replicates of each sample were removed from incubation at 0wk, 3wk, 6wk, 10wk and 24wk and filtered and washed with double-distilled water (dd$H_2O$) onto pre-weighed PVC filter papers (5.0μm pore size, 25mm diameter, Skc Inc). Blank Gambles solution was also washed through five filter papers to control for mass not attributable to the recovered CNTs or fibres. The filtered samples were left to dry protected from dust at room temperature for five days, and then weighed to 0.001mg (Sartorius R180D), and percent recoveries relative to recovery at 0wk were calculated.

In summary, the method used here was as follows: ADD SAMPLES TO GAMBLES SOLUTION → MIX → SONICATE → INCUBATE → FILTER → DRY → WEIGH.

Statistical significance of loss of mass across the time points for each sample type was assessed by one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison post test.

### 2.2.1 Morphological assessment by SEM

After incubation for 0wk and 10wk, samples of each CNT or fibre were filtered on Whatman PC filter membranes (0.2μm pore size, 25mm diameter) and the morphology assessed by SEM (Hitachi S-2600N). EDX (Inca System, Oxford Instruments) linked to the SEM was used to confirm that the major elements present in the samples being viewed were consistent with what was expected.

### 2.2.2 Size quantification by TEM

After incubation for 0wk and 10wk samples of each CNT or fibre were filtered on PVC filter papers (5.0μm pore size, 25mm diameter Skc Inc.) and the size distributions were quantified by TEM. To prepare the samples for TEM, small fragments of filtered material were removed from filter paper with forceps and suspended in 50-100μl ethanol by gentle sonication in a water bath for 20 min. Addition of 1% triton or sonication for longer times did not improve the degree of dispersion. 5μl of each dispersion containing small fragments of material (visible to the naked eye) was pipetted on to a carbon-coated 100-mesh copper grid.

Grids were examined in a Tecnai 12 TEM (FEI, Eindhoven, Netherlands) operating at 120 kV, under a variety of different magnifications. Images were recorded using a MegaView III CCD camera (Olympus) and AnalySiS software.

Measurements were made using Image J (NIH) calibrated via the embedded scale bar.

## *2.3 In vivo* assessment of potential lung inflammation

### 2.3.1 Test samples used

Filtered samples (section 2.2) at 0wk and 10wk were used to investigate the impact of incubation in Gambles solution on inflammogenic potential *in vivo* in mice. These time-points were selected as it was anticipated that, of the samples available in the time-frame for collaborative experimental work, they would be the most likely to show an effect of incubation in Gambles solution, if any effect was present. Note: $CNT_{TANG2}$ was not assessed as it has previously been shown not to elicit an inflammatory response in mice (Poland *et al.* 2008). $CNT_{SPIN}$ was not assessed due to sample characteristics that made it impossible to obtain a good dispersion in 0.5% BSA: saline, therefore we could not be confident of injecting a known mass for *in vivo* evaluation.

### 2.3.2 Animals used in this study

Four female C57Bl/6 mice aged 8 weeks were used for each sample type at each selected time-point, plus three control mice that were injected with vehicle only. All procedures were conducted in accordance with Edinburgh University guidelines.

### 2.3.3 Animal housing conditions

Mice were housed at the University of Edinburgh Biological Research Facility under standard housing conditions of 12h light/ dark cycles and food and water was available *ad libitum*.

### 2.3.4 Injection of fibre samples into mice

The samples (0wk or 10wk residues after filtering, washing and drying) were resuspended in 0.5% BSA in sterile saline at their presumed original mass of 0.5mg/ml by 1h bath sonication (Fisherbrand ultrasonicating water bath, ultrasonic frequency: 40kHz) and 10 sec probe sonication (Bandelin Electronics Status US 70 (Berlin,

Germany): 60% power using pulsing). Based on this presumed 100% recovery of original mass, 50μg each sample or vehicle only, was injected into the mouse peritoneal cavity. The peritoneal cavity is a recognised model for mesothelial exposure [1].

### 2.3.5 Peritoneal lavage and diaphragm harvesting

After 24h or 7d the mice were sacrificed by asphyxiation in 100% $CO_2$ and the peritoneal cavity of each mouse was washed (lavaged) three times with 2ml sterile saline using a 21g needle. The first wash was stored in a chilled 1.5ml Eppendorf tube, and the second and third washes were combined in a conical 15ml falcon tube, both on ice.

Following sacrifice and lavage at the 7d time-point only, the peritoneal cavity was exposed via lateral incisions in the abdominal wall extending to the veterebral column, which was severed below the diaphragm. The ribcage around the diaphragm was then cut from each mouse, taking care not to tear the diaphragm, rinsed by gentle immersion in ice-cold sterile saline and stored in methacarn fixative (60% Methanol, 30% Chloroform, 10% Acetic Acid) at room temperature for approximately five days. The diaphragm was then carefully excised from the surrounding ribcage and stored in 70% ethanol (EtOH) until processed for histology.

### 2.3.6 Cell preparations for *in vitro* assays

To pellet the lavage cells (section 2.3.5), the 1.5ml tubes were centrifuged at 2000 rpm for 5 min at 4°C and the 15ml tubes were centrifuged at 123g for 5 min at 4°C. From the first wash a 1ml aliquot of supernatant was transferred to a fresh 1.5ml Eppendorf tube and stored at -20°C for quantification of Interleukin-6 (IL-6). A further 200μl from the first wash was combined in a fresh 1.5ml Eppendorf tube with 400μl supernatant from the second and third washes and stored at 4°C for quantification of LDH and total protein levels.

#### 2.3.6.1 Total cell count

Total and differential counts were performed to assess infiltration into the peritoneal cavity of immune cells, particularly polymorphonuclear leukocytes (PMNs). The remaining supernatants were discarded and the cell pellet from the second and third washes was resuspended in 500μl 0.01% BSA: saline then combined with the pellet from the first wash, which was completely resuspended by gentle pipetting. An aliquot (10μl) was diluted 1:10 in sterile saline and total cell numbers were counted using a NucleoCounter (ChemoMetec, A/S, Allerød, Denmark) as per standard protocol. Briefly, to the 100μl dilution was added 100μl lysis buffer (ChemoMetic Reagent A) followed by 100μl stabilisation buffer (ChemoMetic Reagent B). The lysed solution was drawn up into a NucleoCounter cassette (Chemometic Nucleocassette™), which was then inserted into the NucleoCounter and the diluted cell count obtained.

#### 2.3.6.2 Differential cell count

After total cell numbers for each sample had been calculated (diluted cell count X10), cyto-centrifugation preparations were prepared for differential cell counting. Glass slides (Thermoscientific) were labelled and placed in a cytospin slide cassette with filter cards (ThermoShandon) and placed in a cytospin centrifuge (Cytospin 4, ThermoShandon). BSA (300μl 0.01% in saline) was added to the cassette and the cell suspension was added at an appropriate volume to obtain ~40 000 cells/slide. The cassettes were centrifuged at 300 rpm for 3 min at room temperature, after which the slides were removed and allowed to dry at room temperature. Once dried, the slides were fixed in 100% methanol then stained with eosin (DiffQuickI, Dade Behring) followed by hematoxylin (Quick-Diff Blue, Reastain). Differential cell counting was performed using light microscopy.

#### 2.3.6.3 Total protein assay

Total protein concentration of the peritoneal lavage fluid was measured using the bicinchoninic acid (BCA) protein assay (Sigma-Aldrich, Poole, UK) in order to assess whether treatment had altered the permeability of the peritoneal cavity. Sample protein concentrations were established by comparison to a BSA standard curve (0 – 1000 μg/ml). The standards and lavage samples were then incubated at 37ºC for 30 min after the addition of the test reagent (1 part copper (II) sulphate solution (4 % w/v) to 50 parts bicinchoninic acid). The absorbance was then read at 570 nm using a Synergy HT microplate reader (BioTek Instruments, Inc. VT, USA) and the sample protein concentration established via extrapolation from the BSA standard curve.

#### 2.3.6.4 IL-6 assay

The concentration of the pro-inflammatory cytokine IL-6 in the peritoneal cavity fluid, measured as an indication of an inflammatory response, was determined by ELISA (IL-6 Duoset, R&D Systems). Sample cytokine concentrations were established by comparison to an IL-6 standard curve (0 – 1000 pg/ml). The samples and standards were added to 96 well plates pre-coated with IL-6 capture antibody and incubated at room temperature for 2 h. Each well was thoroughly washed with 0.05% Tween 20/PBS before incubation with IL-6 detection antibody at room temperature for 2 h. Wells were again washed followed by 20 min incubation with Streptavidin-HRP conjugate. The wash step was repeated before the addition of substrate solution (3, 3', 5, 5' tetramethylbenzidine, Sigma) to each well. Plates were incubated in the dark at room temperature until the colour had sufficiently developed after which the reaction was stopped by the addition of 2N $H_2SO_4$. Absorbance was read at 450 nm using a Synergy HT microplate reader (BioTek Instruments, Inc. VT, USA) and the sample IL-6 concentration established via extrapolation from the IL-6 standard curve.

#### 2.3.6.5 LDH assay

As a measure of cellular membrane damage, the levels of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the peritoneal lavage fluid were assessed using the LDH detection kit (Roche Applied Science). Following addition of the test reagent (diaphorase/$NAD^+$ catalyst mixture diluted in INT and sodium lactate dye solution),

samples were incubated in darkness at room temperature for 30 min after which absorbance was read at 490 nm using a Synergy HT microplate reader (BioTek Instruments, Inc. VT, USA).

### 2.3.7 Quantification of granulomas

The presence of granulomas in the excised diaphragms (section 2.3.5) was quantified as described elsewhere [9] in supplementary information. Briefly, serial images were taken of 4μm diaphragm sections stained with hematoxylin and eosin using QCapture Pro software (Media Cyberbernetics Inc., MD, USA). The images were realigned using Photoshop CS3 (Adobe Systems Inc.) and the total length of each diaphragm along the basement membrane was measured using calibrated Image-Pro Plus software (Media Cyberbernetics Inc., MD, USA) to adjust for size differences between the diaphragms. The area of each detected granuloma was measured, excluding areas of adherent tissue such as liver, connective tissue or lymphatic tissue. The area of granuloma on each diaphragm (in $mm^2$) was expressed per unit length of diaphragm (in mm) to give granuloma area per unit diaphragm length ($mm^2$/mm). Average results were calculated from four separate animals for each treatment and statistical significance was assessed by one-way ANOVA with Tukey's Multiple Comparison post test.

### 2.3.8 Statistics

All data are expressed as the mean ± the standard error of the mean (SE). Statistical tests were performed using GraphPad Prism 5.01 (GraphPad Software, San Diego, USA). Comparisons between two groups were analysed using unpaired t-tests and multiple comparisons were analysed using one-way ANOVA with Tukey's Multiple Comparison post test. For all tests, values of $p<0.05$ were considered statistically significant.

# 3. Results

## 3.1 Durability of test samples

Samples were filtered at 0wk, 3wk, 6wk, 10wk and 24wk, and weighed to 0.001mg (section 2.2). Recovery of the original mass was calculated and expressed as a percentage of the recovery at 0wk to control for potential loss of sample during filtration (Figure 2).

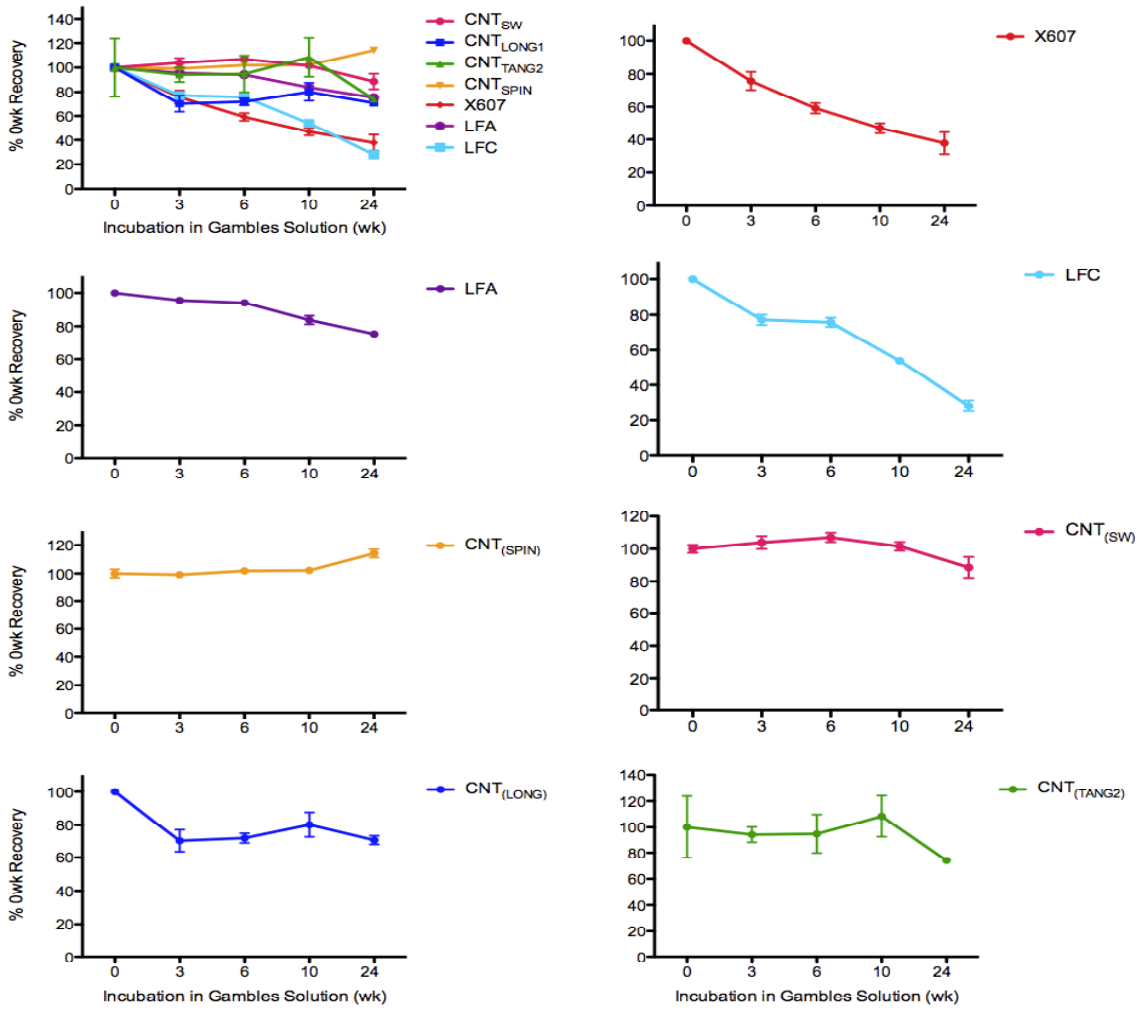


**Figure 2. Sample recoveries after incubation in Gambles solution**

The statistical significance of any differences in mass recovered across the time-points was assessed by one-way ANOVAs with Tukey's Multiple Comparison post tests (Table 3).

**Table 3. Significance of differences in recovered mass following incubation in Gambles solution**

| Sample Type | Incubation Period (wk) | % 0wk Recovery | SE | Significance of Difference from 0wk | Significance of Difference from 3wk | Significance of Difference from 6wk | Significance of Difference from 10wk |
|---|---|---|---|---|---|---|---|
| X607 | 0 | 100 | 2.09 | | | | |
| | 3 | 75.8 | 5.46 | * | | | |
| | 6 | 59.2 | 3.2 | *** | ns | | |
| | 10 | 46.86 | 3.07 | *** | ** | ns | |
| | 24 | 37.82 | 6.6 | *** | *** | * | ns |
| LFA | 0 | 100 | 1.55 | | | | |
| | 3 | 95.53 | 1.48 | ns | | | |
| | 6 | 94.13 | 1.2 | ns | ns | | |
| | 10 | 83.76 | 2.62 | *** | ** | ** | |
| | 24 | 75.43 | 1.33 | *** | *** | *** | * |
| LFC | 0 | 100 | 0.3 | | | | |
| | 3 | 77.26 | 2.93 | *** | | | |
| | 6 | 75.8 | 2.6 | *** | ns | | |
| | 10 | 53.79 | 1.42 | *** | *** | *** | |
| | 24 | 28.23 | 3.03 | *** | *** | *** | *** |
| $CNT_{SPIN}$ | 0 | 100 | 3 | | | | |
| | 3 | 99.14 | 1.23 | ns | | | |
| | 6 | 101.92 | 1.39 | ns | ns | | |
| | 10 | 102.18 | 1.36 | ns | ns | ns | |
| | 24 | 114.18 | 2.91 | ** | ** | ** | ** |
| $CNT_{SW}$ | 0 | 100 | 2.46 | | | | |
| | 3 | 103.92 | 3.94 | ns | | | |
| | 6 | 107.08 | 2.85 | ns | ns | | |
| | 10 | 101.51 | 2.69 | ns | ns | ns | |
| | 24 | 88.68 | 6.4 | ns | ns | * | ns |
| $CNT_{LONG1}$ | 0 | 100 | 1.36 | | | | |
| | 3 | 70.37 | 6.75 | ** | | | |
| | 6 | 71.99 | 3 | ** | ns | | |
| | 10 | 80.19 | 7.41 | ns | ns | ns | |
| | 24 | 70.76 | 2.59 | ** | ns | ns | ns |
| $CNT_{TANG2}$ | 0 | 100 | 23.85 | | | | |
| | 3 | 94.23 | 6.01 | ns | | | |
| | 6 | 94.84 | 14.94 | ns | ns | | |
| | 10 | 108.39 | 15.83 | ns | ns | ns | |
| | 24 | 74.06 | 2.58 | ns | ns | ns | ns |

(*: p<0.05; **: p<0.01; ***: p<0.001; ns: not significant)

These results indicate that, of the control fibres, the non-durable X607 glass fibre lost approximately two-thirds of its mass during the 24wk incubation period. The relatively

durable asbestos fibre, LFA, lost approximately 25% of its mass over the 24wk period, whereas the less durable asbestos fibre, LFC, showed a greater loss of approximately 75% of its original mass during the 24wk incubation.

Of the CNT samples of unknown durability, none had shown a significant loss of mass by 10wk with the exception of $CNT_{LONG1}$, where approximately 30% of mass was lost by 3wk, remaining consistent thereafter up to 24wk. A small but statistically significant increase in mass was observed for the $CNT_{SPIN}$ sample at 24wk. $CNT_{SW}$ and $CNT_{TANG2}$ showed some variation in % recoveries across the time-points but these were generally neither consistent nor statistically significant. However, we note that at 24wk $CNT_{TANG2}$ was recovered at only 74% with little variation (±2.58), therefore it is possible that some mass loss not due to experimental error may have occurred over the longer incubation period. The mass loss at this time-point did not reach statistical significance due to the very large variations in sample recovery at 0wk, reflecting particular difficulties in handling this sample, therefore we note this as a non-significant finding of interest.

Sources of error in sample recoveries possibly include sample preparation by different operators, loss of sample during refreshing of Gambles solution, and loss of sample during filtration, reflecting general difficulties in handling particular samples. We estimated that these sources of error may account for up to 20% of variation in sample recovery and so set ± 20% recovery of the original mass as possibly reflecting experimental error rather than true differences in recoveries, unless the 20% was part of a consistent trend across all time-points. On this basis, despite reaching statistical significance at one or two time-points, we believe the variations in % recoveries for $CNT_{SW}$ and $CNT_{SPIN}$, and perhaps $CNT_{TANG2}$, to reflect experimental error as consistent trends were not evident across all time-points for these samples.

### 3.1.1 Morphology of Filtered Samples by SEM

SEM images of samples that had been incubated for 0wk or 10wk in Gambles solution were taken at 2000 (2.0K) and 5000 (5.0K) magnification (section 2.2.1) and were visually assessed for any obvious changes in morphology. In comparison to fibres incubated for 0wk, X607 fibres that had been incubated for 10wk in Gambles solution showed signs of surface deterioration and etching (Figure 3). No obvious differences in surface structure or fibre morphology for LFA were evident (Figure 4), however apparent thinning as well as fibre splitting was present in LFC fibres incubated for 10wk in comparison to 0wk (Figure 5). In comparison to $CNT_{SPIN}$ samples that had been incubated for 0wk, samples that had been incubated for 10wk were substantially more dispersed and the clumps had developed a more hair-like appearance. However potential differences in surface structures of individual fibres were not assessable due to the difficulty of identifying discrete CNTs (Figure 6). Differences in surface structures of individual $CNT_{SW}$ were also not assessable due to the difficulty of identifying discrete particles but the overall appearance of particle clumps was not altered by the 10wk incubation in Gambles solution (Figure 7). No obvious differences in surface structures or individual CNT morphology were detected between $CNT_{LONG1}$ samples that had been incubated in Gambles solution for 0wk compared to 10wk, however samples that had been incubated for 10wk did appear to show a greater dispersion compared to 0wk (Figure 8). Differences in surface structures of individual $CNT_{TANG2}$ were not

assessable due to the difficulty of identifying discrete particles. No obvious differences in clump morphology were detected, although samples incubated for 10wk in Gambles solution may have been slightly more dispersed compared to samples incubated for 0wk (Figure 9). It must be noted that the 10wk samples were gently bath sonicated for 1h prior to incubation whereas the 0wk samples were not, therefore their generally increased dispersion is most likely in part attributable to this sonication as well as to the 10wk incubation period. However, these images have still been included for comparison.

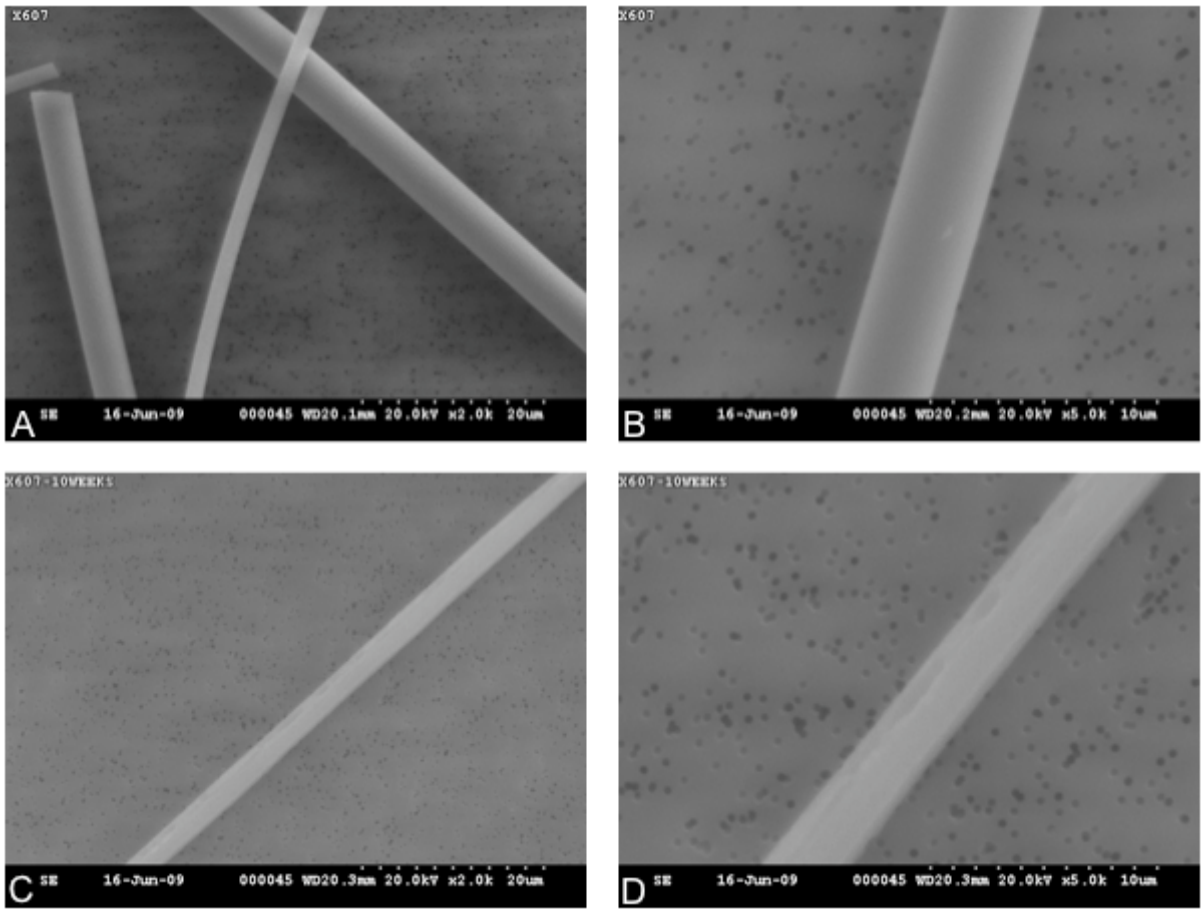


**Figure 3. X607 glass wool fibres under SEM at A. 0wk 2.0K magnification; B. 0wk 5.0K magnification; C. 10wk 2.0K magnification; 10wk 5.0K magnification.**

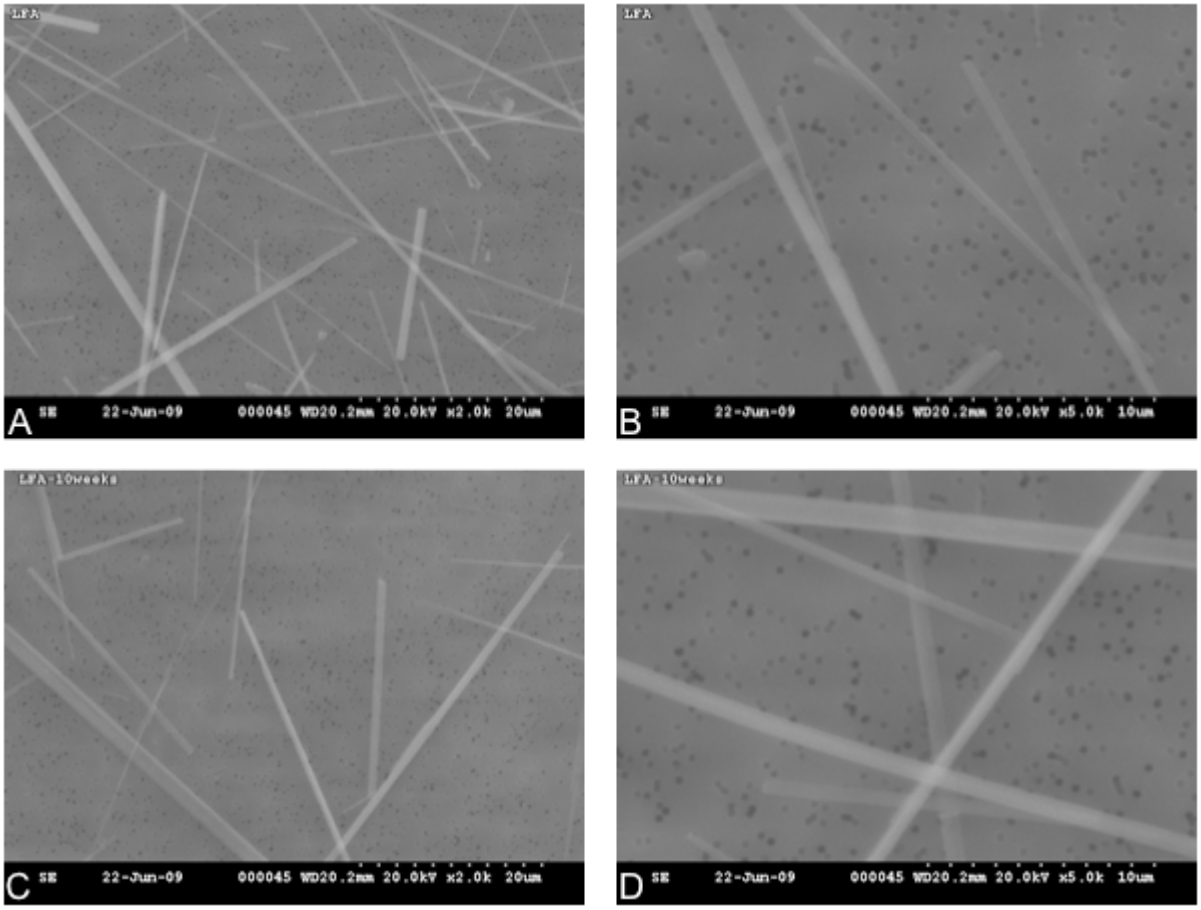


**Figure 4. LFA asbestos fibres under SEM at A. 0wk 2.0K magnification; B. 0wk 5.0K magnification; C. 10wk 2.0K magnification; D. 10wk 5.0K magnification.**

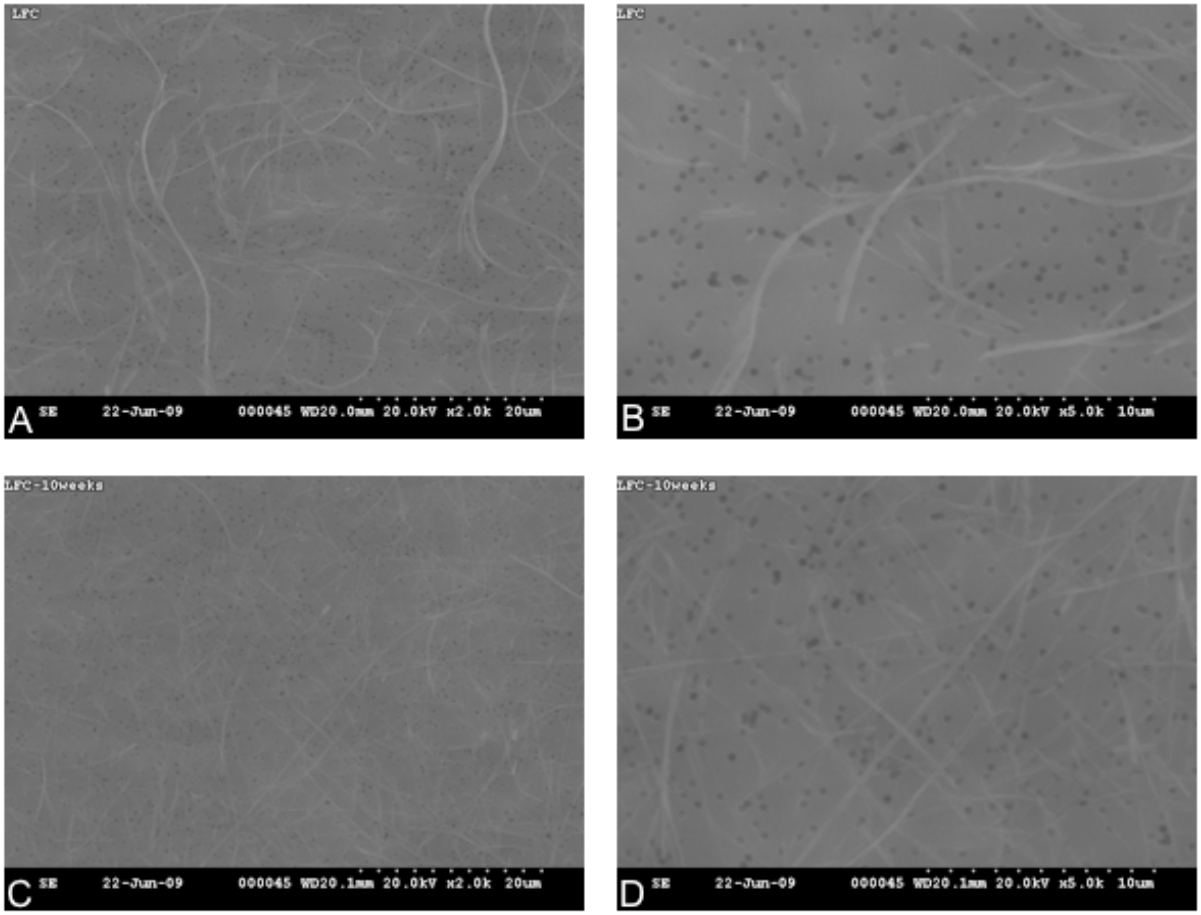


**Figure 5. LFC asbestos fibres under SEM at A. 0wk 2.0K magnification; B. 0wk 5.0K magnification; C. 10wk 2.0K magnification; D. 10wk 5.0K magnification.**

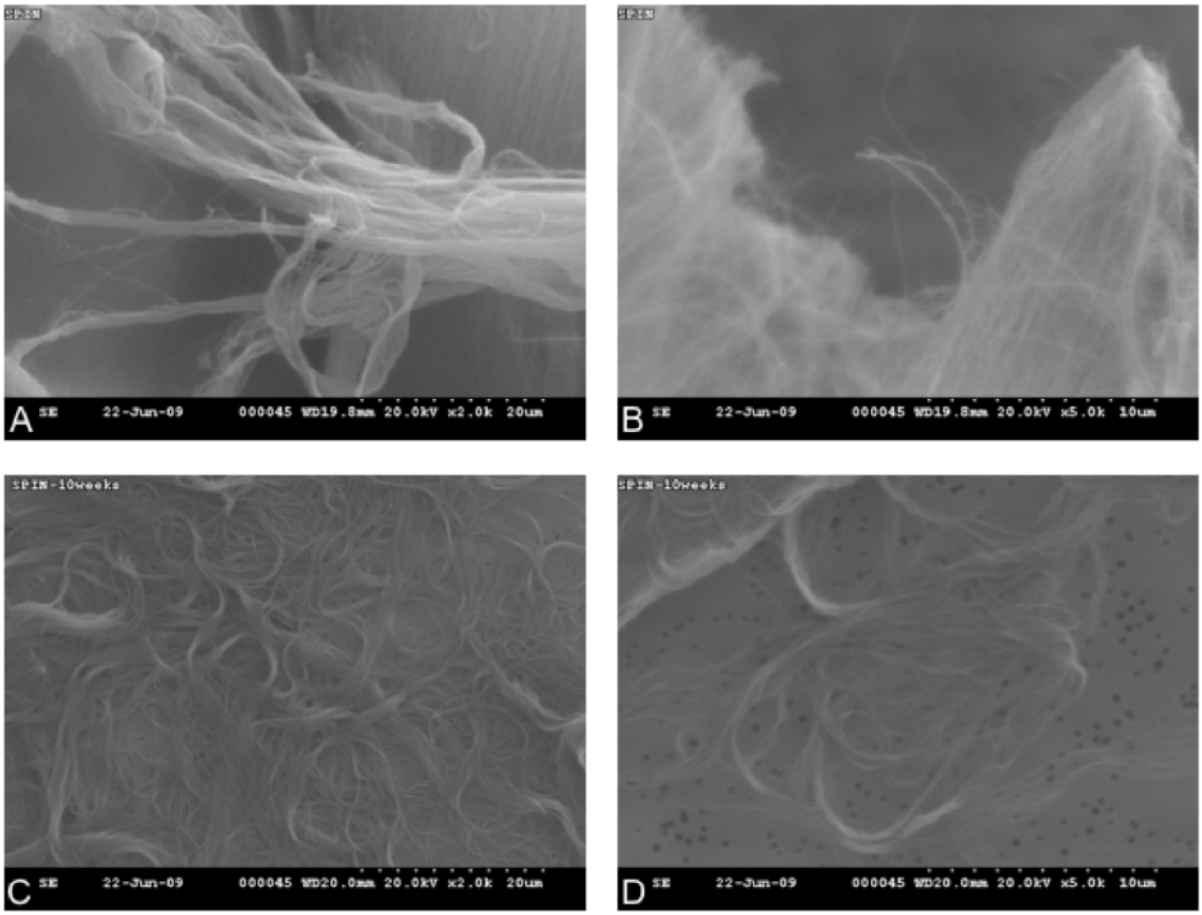


**Figure 6. $CNT_{SPIN}$ under SEM at A. 0wk 2.0K magnification; B. 0wk 5.0K magnification; C. 10wk 2.0K magnification; D. 10wk 5.0K magnification.**

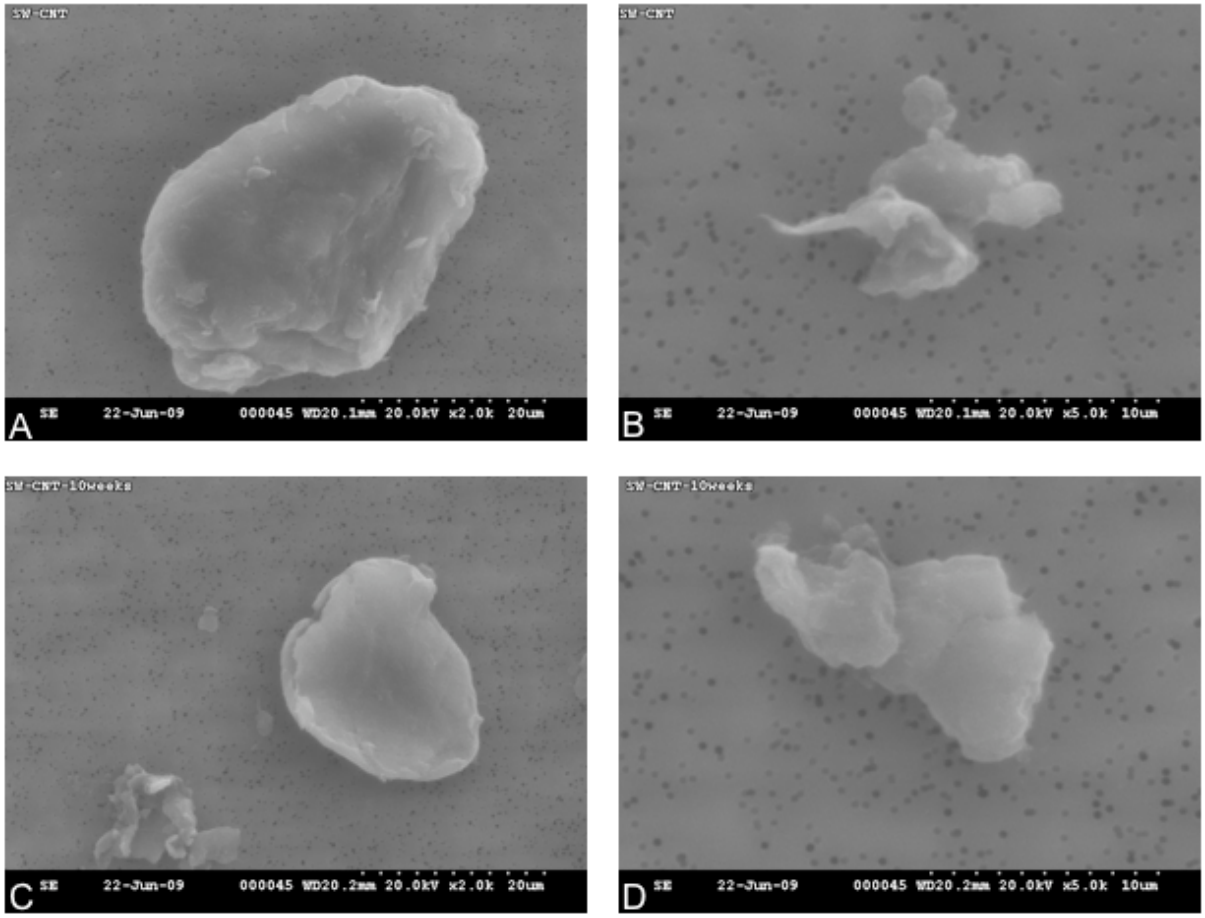


**Figure 7. $CNT_{SW}$ under SEM at A. 0wk 2.0K magnification; B. 0wk 5.0K magnification; C. 10wk 2.0K magnification; D. 10wk 5.0K magnification.**

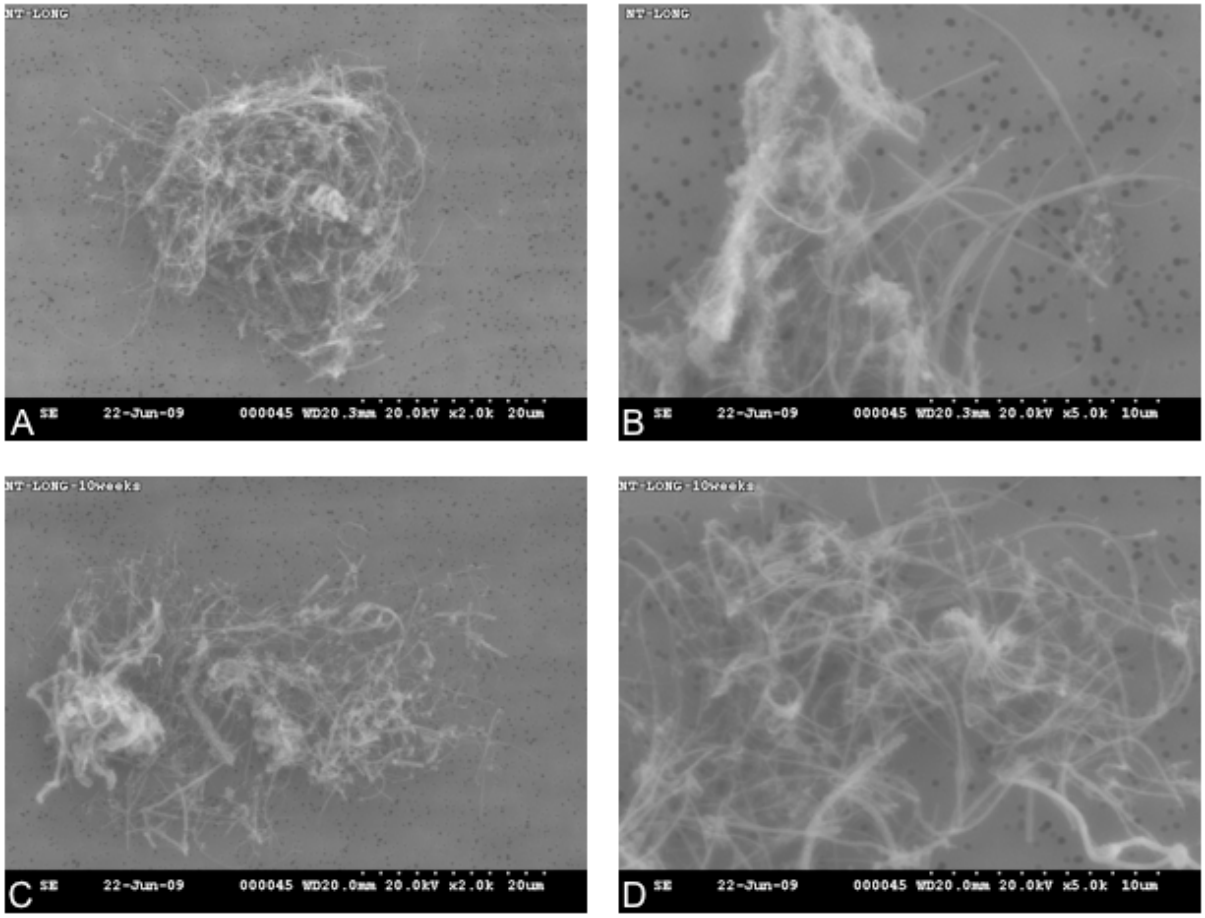


**Figure 8. $CNT_{LONG1}$ under SEM at A. 0wk 2.0K magnification; B. 0wk 5.0K magnification; C. 10wk 2.0K magnification; D. 10wk 5.0K magnification.**

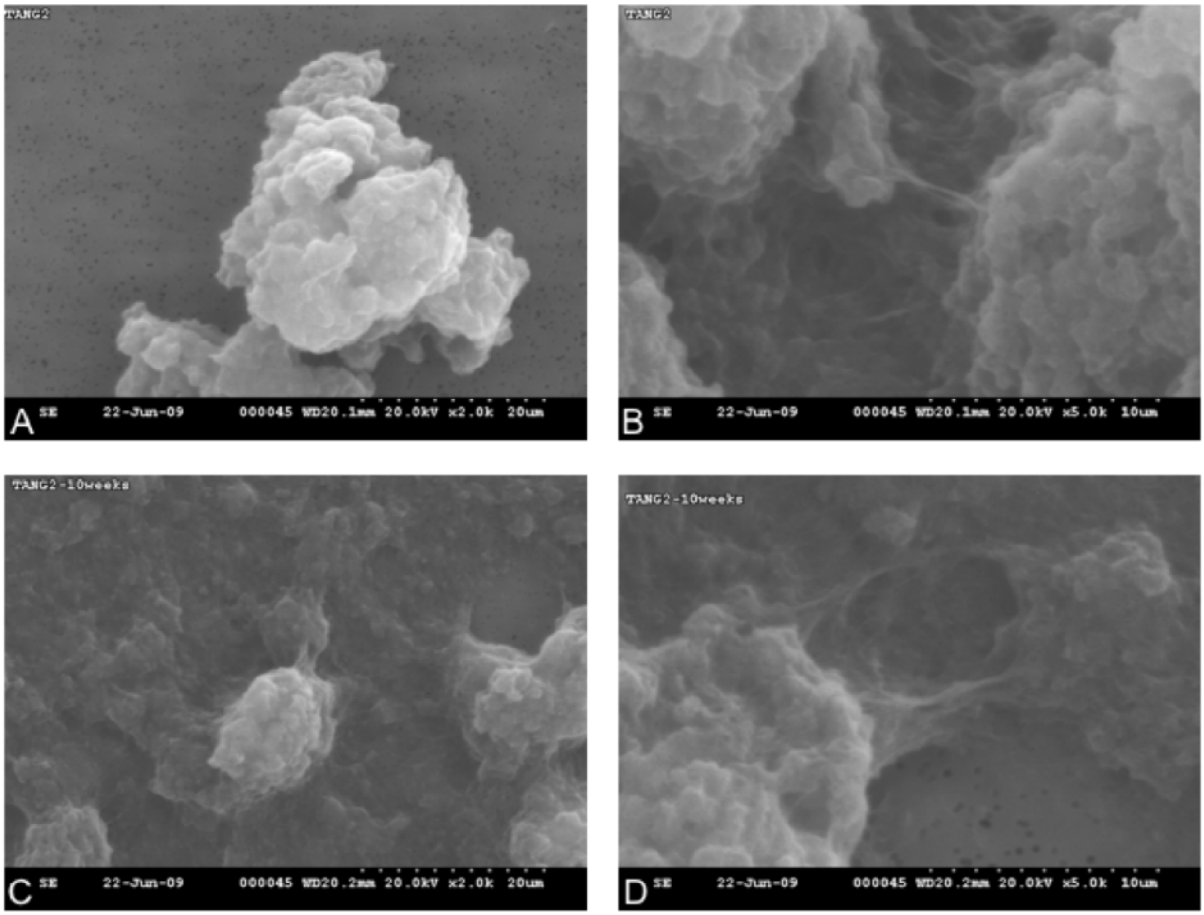


**Figure 9. $CNT_{TANG2}$ under SEM at A. 0wk 2.0K magnification; B. 0wk 5.0K magnification; C. 10wk 2.0K magnification; D. 10wk 5.0K magnification.**

### 3.1.2 Sample size quantification by TEM

TEM images of samples that had been incubated for 0wk or 10wk in Gambles solution were taken at various magnifications (section 2.2.2) and the lengths and widths of 100 CNTs or fibres for each sample were measured using Image J (NIH) calibrated software. Statististically significant differences (two-way unpaired t-test) between 0wk or incubated samples (3wk or 10wk) are denoted by asterisks (Table 4). Representative TEM images for each sample are shown in Figures 10-16. Here it should again be noted that the 10wk samples received 1h of gentle bath sonication prior to incubation whereas the 0wk samples did not, therefore this must be taken into account when comparing the images with regard to dispersion.

X607 samples were the largest fibres assessed here, with widths in the micron range and average lengths markedly greater than 20µm. These characteristics remained, even after 10wk incubation in Gambles solution, however the average width decreased from 3.5 to 2µm and the proportion of long fibres also decreased, although a high percentage remained. It can also be seen from Figure 10 that what appeared to be

small remnants of the fibres flecked the TEM images when viewed under high magnification (Figure 10F).

LFA showed an apparent increase in average fibre length and width, although not statistically significant due to the large standard deviations. The proportion of long fibres also apparently increased as a result of the incubation, which is difficult to explain. It is possible that the loss of mass at the last two time-points for this sample reflect the loss of smaller fibres, leaving, on average, a greater proportion of longer fibres remaining in the recovered 75%. This would potentially bias the average length to the longer end of the size distribution. The fibres did not show any morphological differences under TEM (Figure 11).

LFC showed no difference in average fibre width with incubation but did show a marked decrease in length. However, it was noted that at 0wk the LFC fibres comprised a mixture of individual fibrils as well as fibrils aligned into rope-like structures. All were measured in the 0wk sample however at 10wk only smaller individual fibrils remained, leaving the average length biased to the small. Given this sample also showed a marked loss of mass, and is known to be non-durable, it is probable that the loss of length also reflects fibre shortening in addition to the noted loss of large fibre bundles. Fibres were also sparser when viewed by TEM (Figure 12).

$CNT_{SW}$ did not show an alteration in average length or width arising from incubation in Gambles solution and also showed no morphological changes under TEM (Figure 13), with the majority of fibres forming large clumps in which the presence of individual fibres could generally only be ascertained at the edges.

Lengths for $CNT_{SPIN}$ could not be determined due to their very long, hair-like nature, making the starts and ends of individual tubes virtually impossible to identify. However, the average width increased very slightly after 10wk incubation in Gambles solution. Under TEM some contaminating material, perhaps amorphous carbon, appears in the 0wk sample but was present to a lesser degree in the 10wk sample, suggesting it may have been lost during incubation (Figure 14). If so, the weight of this material relative to the carbon nanotubes would be very small as no weight loss was measured after 10wk incubation.

$CNT_{LONG1}$ samples that had been incubated in Gambles solution for 3wk and 10wk showed small decreases in average fibre lengths as well as decreases in the proportion of long fibres present compared to samples incubated for 0wk, with approximately 50% fewer fibres >15μm and >20μm in samples incubated for 10wk compared to the percentages in samples incubated for 0wk. However, despite the proportion of long fibres decreasing with incubation, the average fibre length remained higher than 10μm, indicating that a substantial fraction of fibres longer than this remained. TEM images show that many fibres in this sample contained what may be amorphous carbon (Figure 15B, C, E, F) and a large number of what appear to be curled up CNTs decorating the straighter fibres.

The lengths for $CNT_{TANG2}$ were not able to be determined due to the “tangled” nature of the fibres and subsequent difficulties identifying discrete tubes from start to end, but

the average width did not change as a result of the incubation in Gambles solution and morphology did not alter as a result of incubation when viewed by TEM (Figure 16).

**Table 4. Sample lengths and widths after 0wk[1] or 10wk incubation in Gambles solution**

| Sample | Incubation Period (wk) | Width (nm) ± SE | Length (μm) | % Fibres >15μm | % Fibres >20μm |
|---|---|---|---|---|---|
| X607 | 0 | 3500 ± 2000 | 123 ± 69 | 98 | 94 |
| | 10 | 2100 ± 1400* | 76 ± 46* | 95 | 88 |
| LFA | 0 | 550 ± 390 | 34 ± 36 | 63 | 54 |
| | 10 | 820 ± 490 | 56 ± 52 | 79 | 74 |
| LFC | 0 | 42 ± 12 | 11 ± 12 | 26 | 20 |
| | 10 | 43 ± 13 | 1.9 ± 1.6* | 0 | 0 |
| $CNT_{SW}$ | 0 | 5 ± 2 | 4 ± 2 | 0 | 0 |
| | 10 | 5 ± 2 | 3 ± 2 | 0 | 0 |
| $CNT_{SPIN}$ | 0 | 9 ± 3 | NAs | NAs | NAs |
| | 10 | 14 ± 3* | NAs | NAs | NAs |
| $CNT_{LONG1}$ | 0 | 64 ± 16 | 12 ± 6 | 30 | 10 |
| | 3[2] | 65 ± 20 | 11 ± 6* | 18 | 8 |
| | 10 | 63 ± 28 | 11 ± 5* | 13 | 4 |
| $CNT_{TANG2}$ | 0 | 10 ± 5 | NAs | NAs | NAs |
| | 10 | 10 ± 4 | NAs | NAs | NAs |

NAs: not assessable

[1] Data at 0wk are the same as in Table 2

[2] Lengths and widths were determined at 3wk for $CNT_{LONG1}$ to confirm the shortening seen in the 10wk sample.

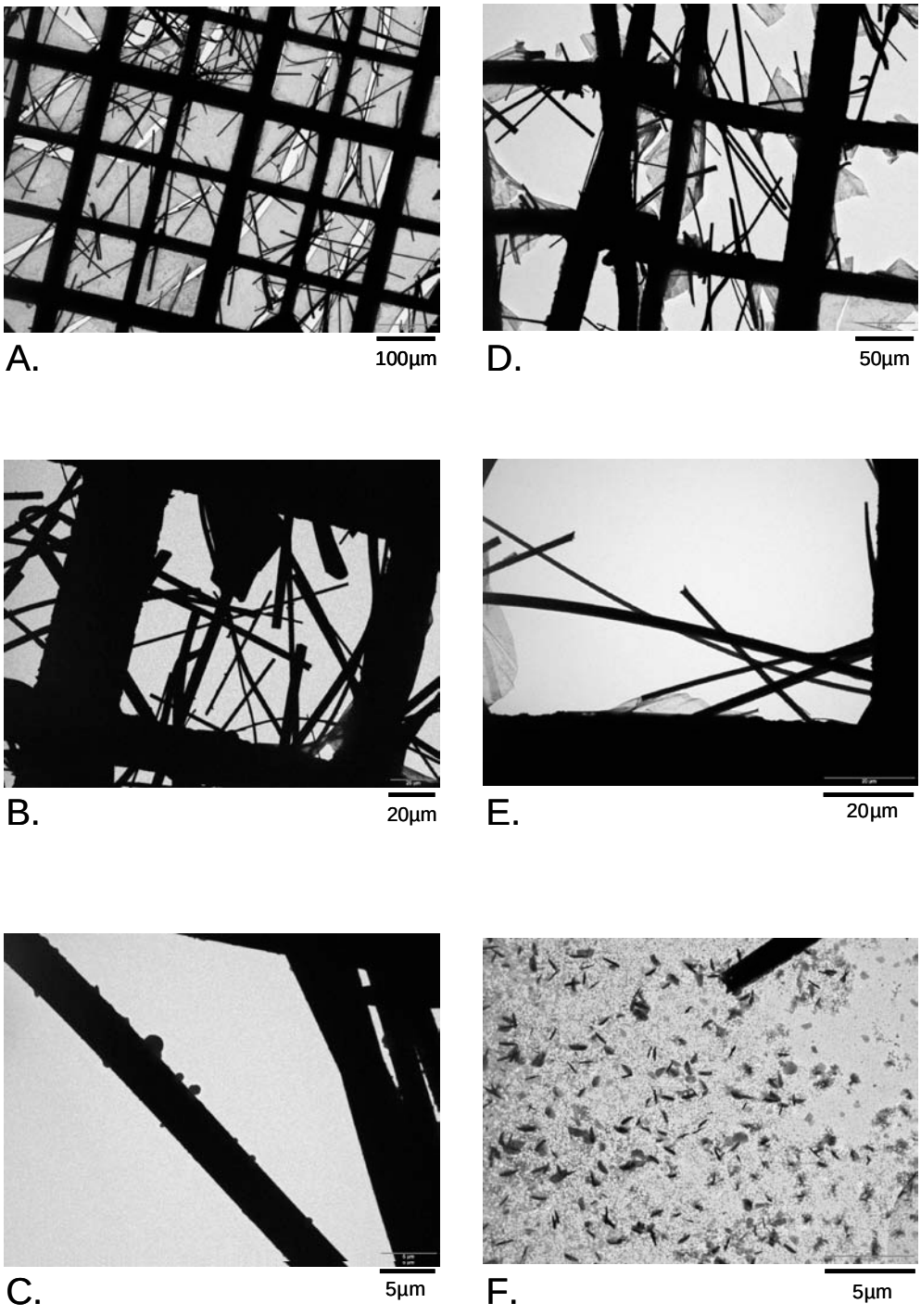


**Figure 10. X607 glass wool fibres under TEM after 0wk (A 145X magnification; B 560X magnification; C 2700X magnification) and 10wk (D 290X magnification; E 1100X magnification; F 4400X magnification) incubation**

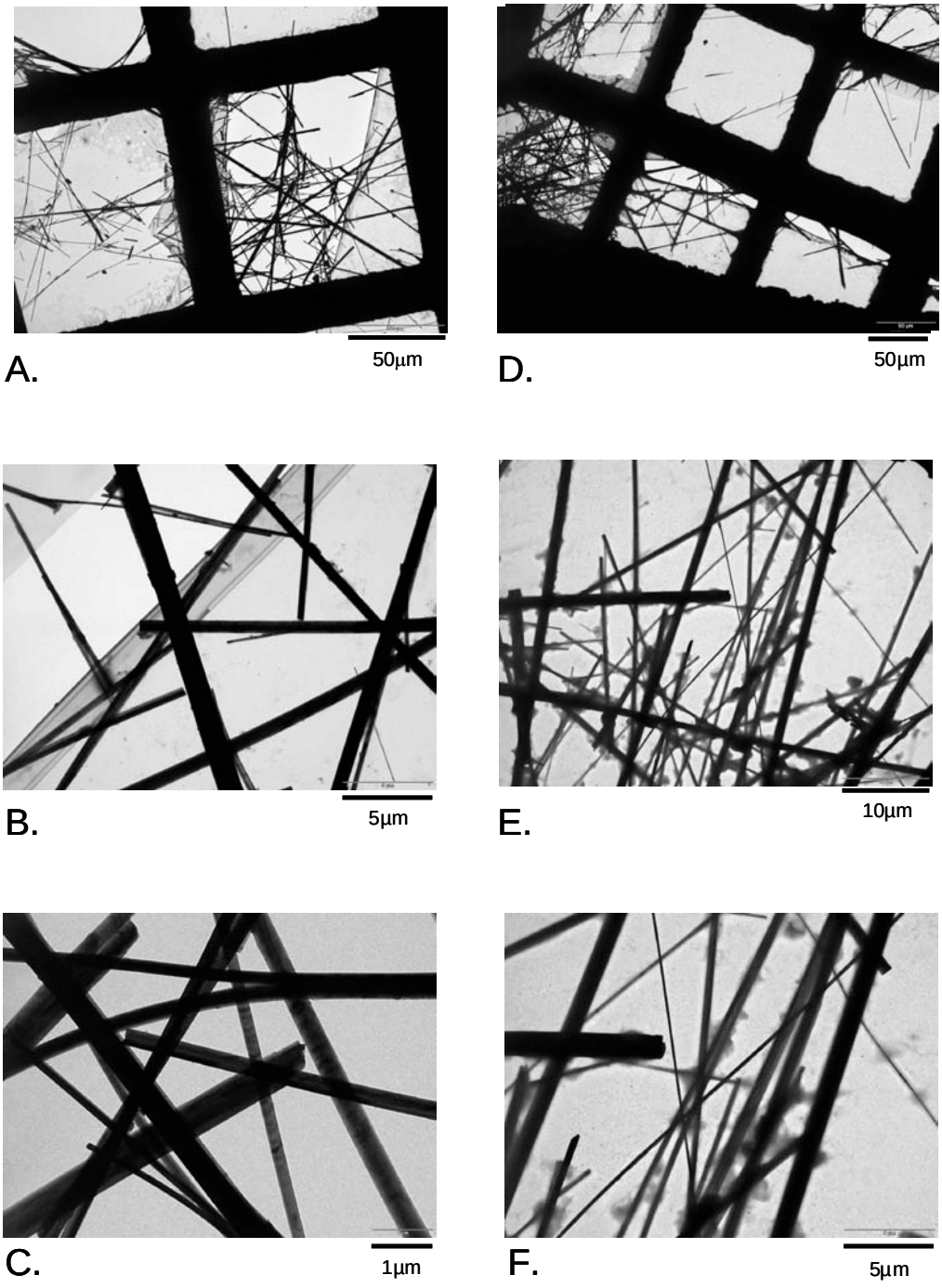


**Figure 11 LFA asbestos fibres under TEM after 0wk (A 470X magnification; B 4400X magnification; C 21000X magnification), and 10wk (D 290X magnification; E 2100X magnification; F 4400X magnification) incubation**

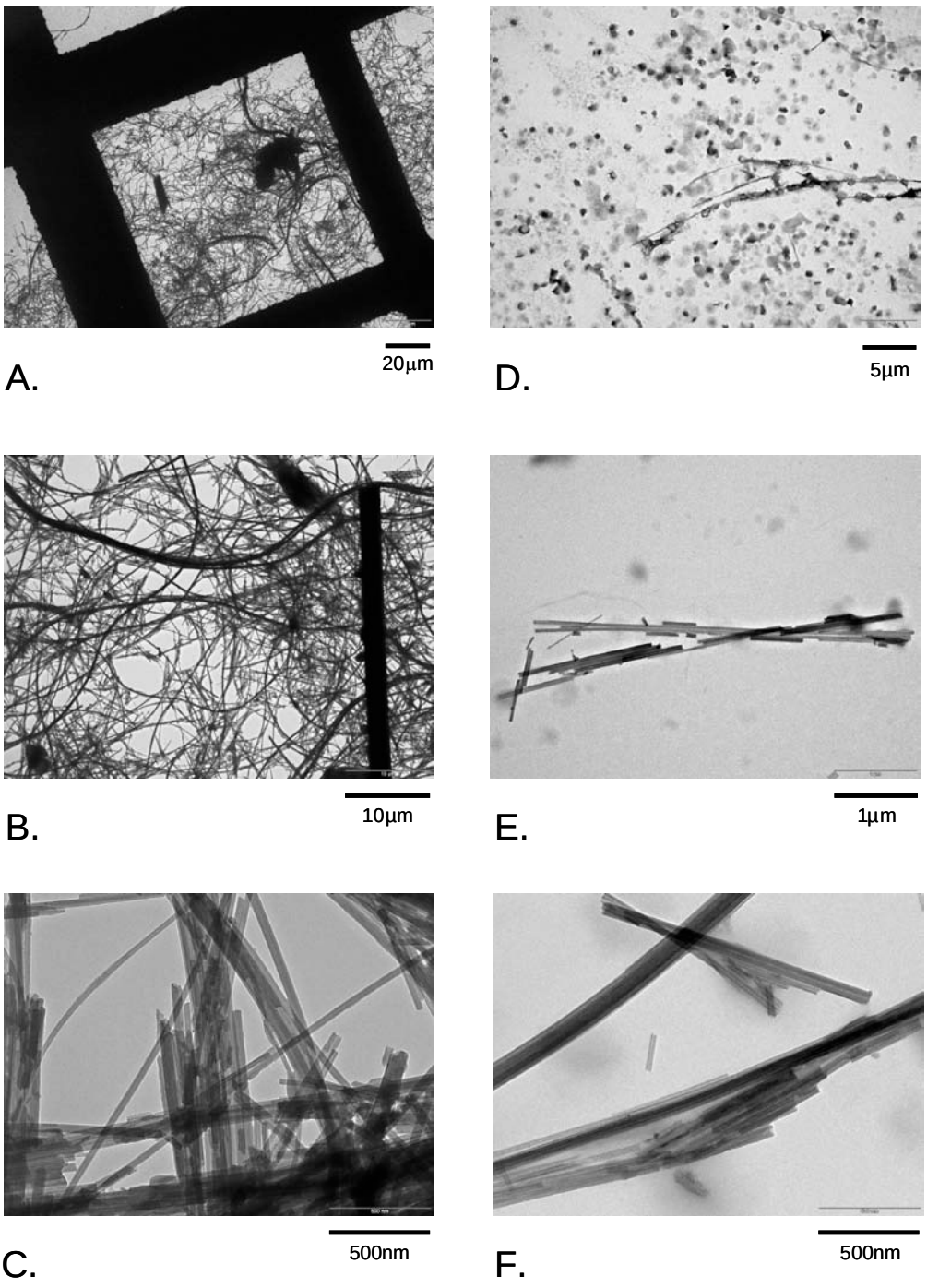


**Figure 12. LFC asbestos fibres under TEM after 0wk (A 560X magnification; B 2100X magnification; C 52000X magnification), and 10wk (D 2700X magnification; E 21000 magnification; F 52000X magnification) incubation**

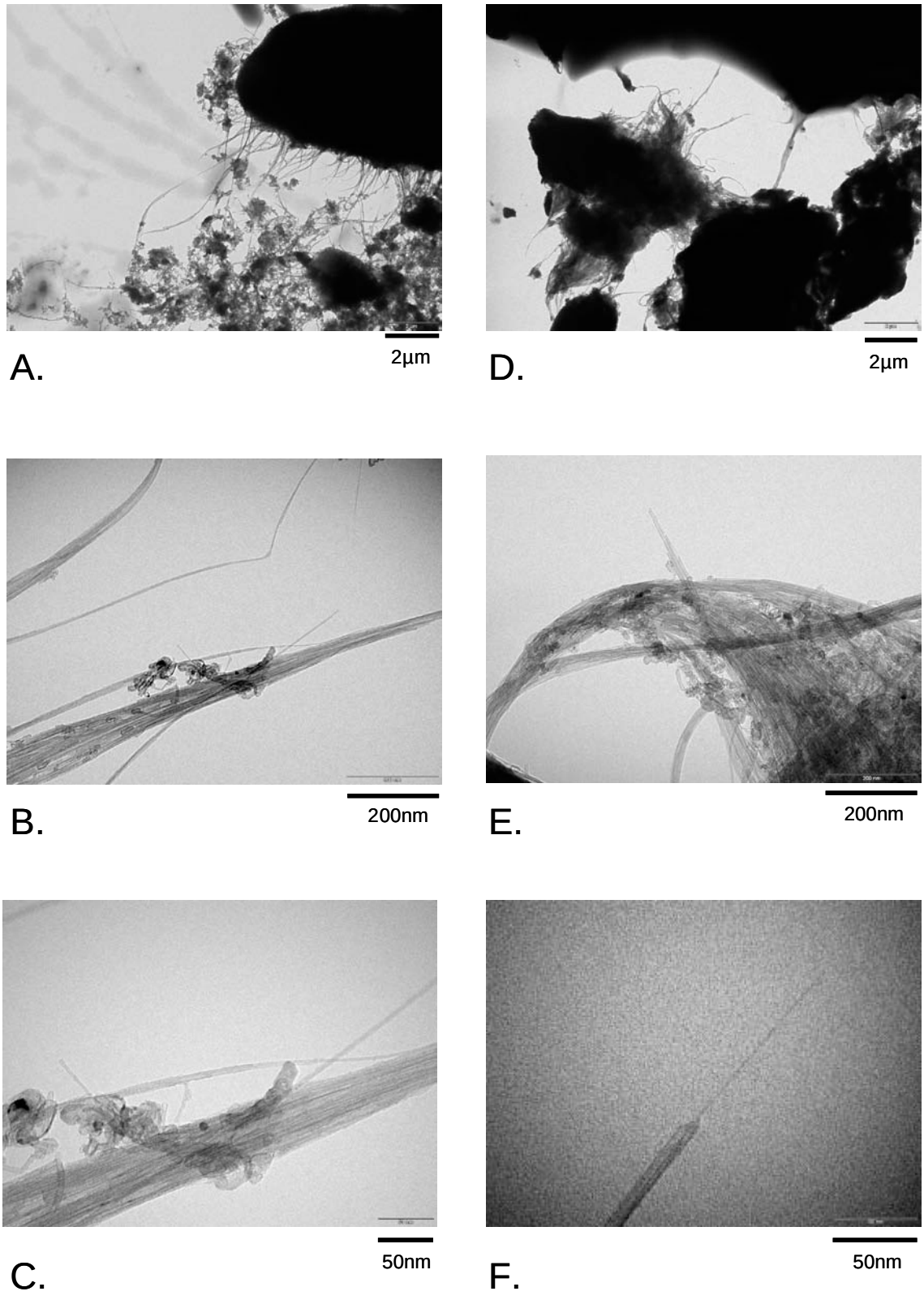


**Figure 13. $CNT_{SW}$ fibres under TEM after 0wk (A 6500X magnification; B 110000X magnification; C 265000X magnification) and 10wk (D 6500X magnification; E 110000X magnification; F 400000X magnification) incubation**

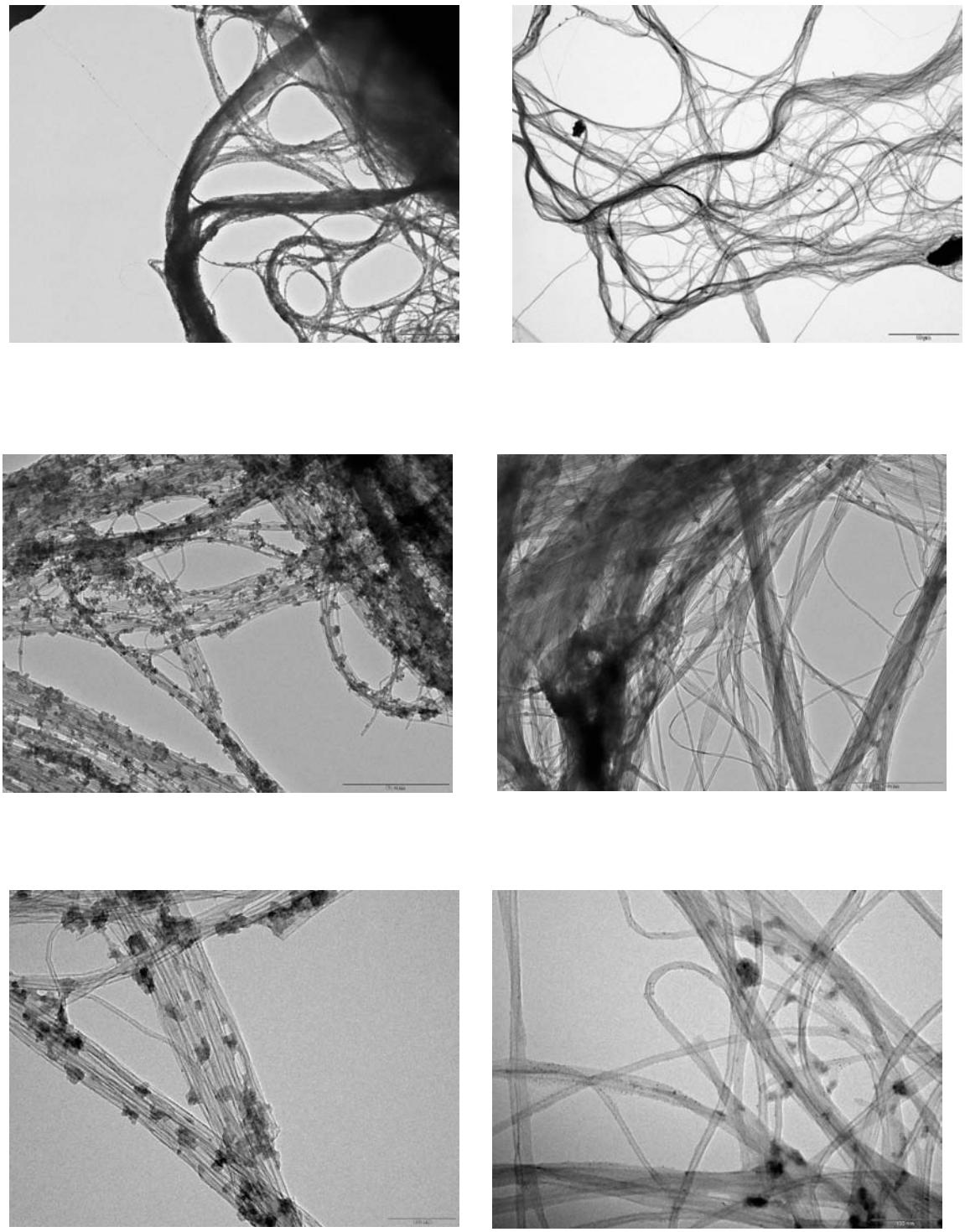

**Figure 14. $CNT_{SPIN}$ fibres under TEM after 0wk (A 6500X magnification; B 52000X magnification; C 150000X magnification) and 10wk (D 1650X magnification; E 52000X magnification; F 150000X magnification) incubation**

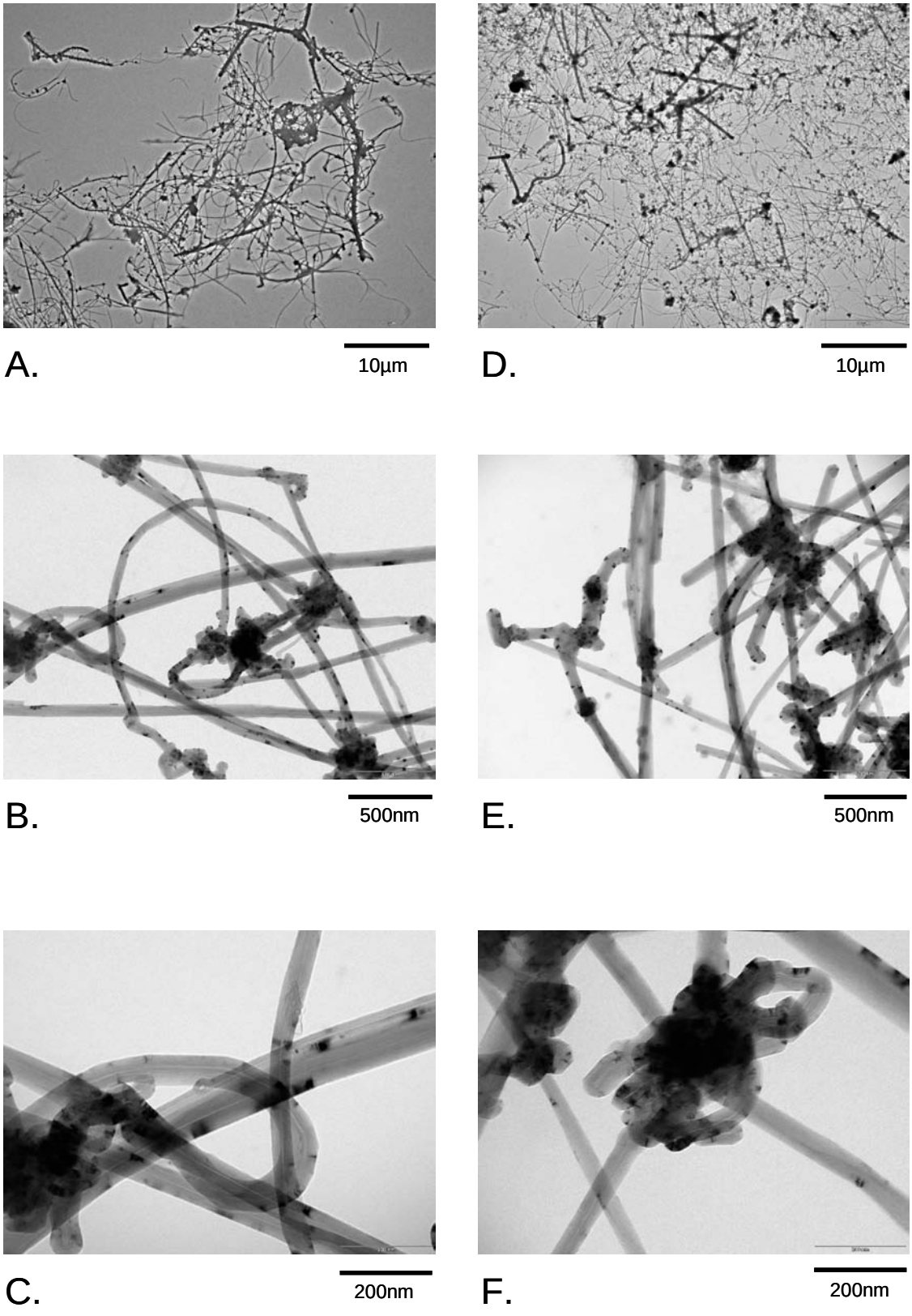


**Figure 15. $CNT_{LONG1}$ fibres under TEM after 0wk (A 2100X magnification; B 42000X magnification; C 110000X magnification) and 10wk (D 2100X magnification; E 42000X magnification; F 110000X magnification) incubation**

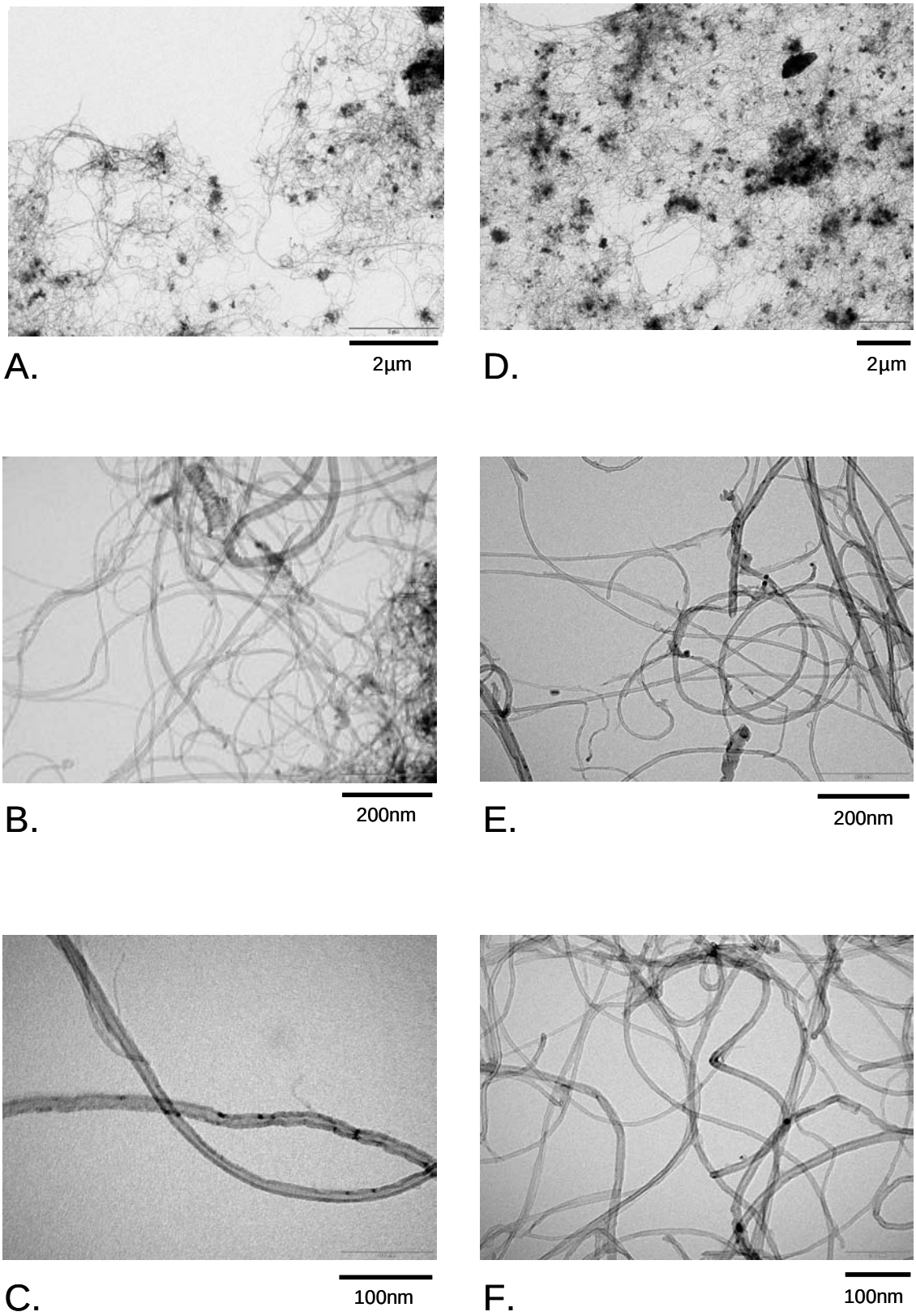


**Figure 16. $CNT_{TANG2}$ fibres under TEM after 0wk (A 11000X magnification; B 110000X magnification; C 220000X magnification) and 10wk (D 6500X magnification; E 110000X magnification; F 150000X magnification) incubation**

## 3.2 *In vivo* inflammogenic response to test samples

Filtered samples that had been incubated in Gambles solution for 0wk or 10wk were resuspended in 0.5% BSA:saline and a presumed mass of 50μg was injected into the peritoneal cavity of female C57BL/6 mice (section 2.3.4). Mice were sacrificed 24h or 7d post-injection and the peritoneal cavity was washed and lavage fluid collected. A number of *in vitro* assays used to identify the presence of an acute inflammatory response were performed here: total and differential cell counting identified possible infiltration of PMNs into the peritoneal cavity in response to the treatments; measurement of the inflammatory cytokine, IL-6, was indicative of the release of inflammatory cytokines; measurement of total protein was indicative of increased permeability in the peritoneal cavity; and measurement of LDH was indicative of damage to cellular membranes. In addition, the development of granulomas was assessed. $CNT_{TANG2}$ and $CNT_{SPIN}$ were excluded from *in vivo* analysis because the former had previously been shown to be non-pathogenic in a similar study [9] and the latter was not able to be dispersed well enough to inject a reliable amount of sample into the mice. A summary of the statistically significant results for the durability and *in vivo* assays are summarized in Table 5. A complete description of the results for each assay can be found in Appendix I.

It can be seen from these results that, of the known non-durable fibres, X607 did not elicit a significant inflammatory response, regardless of incubation, whereas LFC that had been incubated in Gambles solution for 0wk elicited an acute inflammatory response that had subsequently subsided by 7d alongside the development of a granuloma response. In contrast, LFC that had been incubated for 10wk showed fibre shortening and had lost some of the inflammogenic potential of the 0wk fibres. The known durable fibre, LFA, showed some loss of mass but no fibre shortening, and elicited an acute inflammatory response in mice in addition to the development of granulomas by 7d, regardless of incubation, suggesting that long-term incubation did not alter its pathogenicity. Of the CNTs, $CNT_{SW}$ showed no fibre changes with incubation and did not induce an inflammatory response. $CNT_{LONG1}$ showed loss of mass and a decreased proportion of longer fibres (although average length was only slightly decreased) with long-term incubation in Gambles solution. $CNT_{LONG1}$ 0wk induced an acute inflammatory response in mice that did not completely subside by 7d post-injection and also induced the strongest granuloma response of all fibres investigated here on a *mass* basis. In contrast, $CNT_{LONG1}$ 10wk, which had shown loss of mass and fibre shortening in comparison to 0wk, was less pathogenic in mice, inducing a weaker inflammatory and granuloma responses.

**Table 5. Summary[3] of durability, acute *in vivo* inflammatory response and 7d *in vivo* inflammatory response compared to vehicle only**

| | | Fibre Changes | | Acute Inflammatory Response (24h) | | | | | 7d Inflammatory Response | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Loss of Mass | Fibre Shortening | Total Cell | PMN Infiltration | Total Protein | IL-6 | LDH | Total Cell | PMN Infiltration | Total Protein | IL-6 | LDH | Granuloma Response |
| X607 | 0wk | N/A | N/A | - | - | - | - | - | - | - | - | - | - | - |
| | 10wk | + | + | - | - | - | - | - | - | - | - | - | - | - |
| LFA | 0wk | N/A | N/A | + | + | - | - | - | - | - | - | - | - | Yes (ns) |
| | 10wk | + | - | + | + | - | - | - | - | - | - | - | - | Yes (ns) |
| LFC | 0wk | N/A | N/A | + | + | + | - | - | - | - | - | - | - | Yes (ns) |
| | 10wk | + | + | - | - | - | - | - | - | - | - | - | - | - |
| $CNT_{SW}$ | 0wk | N/A | N/A | - | - | - | - | - | - | - | - | - | - | - |
| | 10wk | - | - | - | - | - | - | - | - | - | - | - | - | - |
| $CNT_{LONG1}$ | 0wk | N/A | N/A | + | + | + | + | - | - | - | + | + | + | Yes (s) |
| | 10wk | + | + | - | - | - | - | - | - | - | - | - | - | - |

(+ = statistically significant ($p$<0.-05); - = not statistically significant; (ns) = not significant; (s) = significant; N/A: not applicable)

[3] The experiments and data used to compile this summary are given in Appendix I

## 4. Discussion

The first part of these experiments was designed to determine the durability of a range of CNTs after long-term incubation in simulated lung fluid in comparison to fibres of known durability. The glass wool fibre, X607, and asbestos fibre, LFC, both showed an approximate 75% loss of mass over the 24wk incubation period in Gambles solution, with their apparent dissolution consistent with their known non-durable natures [10-12]. This loss of mass was confirmed by SEM and TEM, which showed surface etching and splitting, and fibre thinning and shortening, respectively. As a durable fibre, LFA was not expected to show a substantial loss of mass and indeed after 24wk in Gambles solution ~75% of the fibres were recovered, and visualisation by SEM and TEM revealed no obvious morphological changes or fibre shortening.

Given the control fibres behaved more or less as expected, it is thus interesting that not all types of CNTs assessed here showed the biodurabilty that was anticipated given their essentially graphenic nature [5]. Rather, $CNT_{SW}$ and $CNT_{SPIN}$ were recovered at ~100% across the time points and showed no morphological alterations under SEM or TEM. $CNT_{TANG2}$ may have shown a slow loss of mass resulting in only 74% recovery at 24wk, however this did not reach statistical significance due to the high variation in the recovery of this sample at 0wk, and no alterations in fibre morphology were observed under SEM or TEM. In contrast, $CNT_{LONG1}$ showed a sharp loss of ~30% mass at 3wk in Gambles solution, which remained consistent at subsequent time points, although as 3wk was the first interrogation time the mass loss may have occurred earlier. One explanation for this could be that there may have been some soluble material within the $CNT_{LONG1}$ sample that dissolved relatively quickly, leaving the more durable 70% remaining. However, examination under TEM of $CNT_{LONG1}$ that had been incubated for 3 wk and 10wk compared to 0wk showed that a substantial number of fibres had also undergone shortening, with the percentage fibres >20μm decreasing from 10% to 8% by 3wk incubation and decreasing further to 4% by 10wk, and the percentage fibres >15μm decreasing from 30% to 18% by 3wk and further to 13% by 10 wk. These results therefore suggest that even if the observed loss of mass was in part attributable to the dissolution of a potentially soluble component of the $CNT_{LONG1}$ material, this was accompanied by apparent fibre shortening.

It is possible that two different mechanisms contributed to this shortening: mechanical breakdown from sonication, and dissolution in Gambles solution. Although we cannot exclude the former, particularly as we did not sonicate the 0wk samples prior to filtration, the continued decrease in percent long fibres in the 10wk samples compared to 3wk samples suggests that dissolution was a contributing factor. In order to increase confidence that the 1h gentle sonication of the incubated fibres did not mechanically shear the $CNT_{LONG1}$ sample as described elsewhere using different CNTs and under different conditions [13], we conducted a small follow-up experiment (data not shown) whereby $CNT_{LONG1}$ was in quadruplicate either sonicated in Gambles solution for 1h under conditions identical to the main experiment, then filtered, dried and weighed, or washed in Gambles solution and immediately filtered, dried and weighed, replicating the conditions by which we produced our original 0wk samples. We found no mass loss resulting from the 1h sonication, and that the percent recoveries for 0h or 1h sonicated samples were not different from each other. We then measured the fibre lengths for

both conditions and again found no statistical difference in either average fibre length or proportion of long fibres. Additionally, we examined the filtrates from the original 0wk and 10wk samples by TEM, as well as from the new 0h and 1h sonicated samples. Only the original 10wk samples contained CNT debris (fragments approximately 500nm in length). When taken together, the weight of evidence supports the view that the changes in $CNT_{LONG1}$ morphology and pathogenicity reported here result largely from incubation in the Gambles solution rather than mechanically from the 1h sonication. Further support for this view is indirectly provided elsewhere [9], where the same $CNT_{LONG1}$ sample as used here retained pathogenicity despite 2h bath sonication.

Fibre shortening would potentially impact the long-term pathogenicity of this particular type of CNT in biological systems. It is therefore of relevance that a similar CNT breakdown has been described elsewhere, with chemical modification and cleavage of MWCNTs in rat lungs over time [14]. However, it is important to note that two of the other types of CNTs assessed here did not show loss of mass or change in morphology and a third, whilst showing a possible loss of mass at 24wk, showed no morphological changes, and therefore the fibre shortening and loss of mass seen in the $CNT_{LONG1}$ sample cannot be generalised across all types of CNTs. Indeed, following intratracheal instillation in rats, MWCNTs were found to be not, or only slowly, cleared from the lung, with 81.2% of the dose recovered from the lungs 60 days post-instillation compared to only 36% recovered in rats instilled with the same type of CNT only in ground up form [15], although in that study the CNTs had an average length of 6μm and therefore might be expected to be cleared by macrophages over time. Muller's results suggested that in the lung environment particulate CNTs were cleared at a faster rate than fibrous CNTs, but their presence after 60 days was also indicative of sample biopersistence in the lung environment. By the equivalent time in our study we had observed fibre shortening and loss of mass in the $CNT_{LONG1}$ sample, although not for the other three CNT samples, except for the possible loss of mass at 24wk for $CNT_{TANG2}$. Therefore, CNT durability might be sample-specific.

The aim of the second part of this study was to assess the impact of long-term incubation in Gambles solution on the pathogenicity of CNTs compared to fibres of known durability when subsequently injected into the peritoneal cavity of mice for 24h or 7d. We found that when the CNTs had been incubated in Gambles solution for 0wk (i.e. washed and filtered immediately) the pathogenicities of LFA and $CNT_{LONG1}$ were largely as previously reported [9], and our results overall were consistent with what might be predicted by the fibre pathogenicity paradigm [5]. The $CNT_{SW}$ samples did not induce an inflammatory response, which might be anticipated given the propensity we found for this particular type of CNT to form tightly agglomerated bundles. Schipper et al. (2008) reported that their functionalised SWCNTs did not form aggregates in water or biological media and also did not induce an inflammatory response when injected into the bloodstream of mice, although the presence of SWCNTs could be observed in liver and spleen macrophages up to four months post-injection [16]. The lack of pathogenicity of the $CNT_{SW}$ sample here, however, may not appear to be consistent with other studies, where inflammatory and granuloma responses were observed in mice and rats that had received doses of SWCNTs via intratracheal instillation [17, 18]. However, the mouse peritoneal cavity, in contrast to the lungs, is not responsive to compact particles or short fibres, only to long fibres, and so no effect was anticipated.

Following lung deposition the fibrotic response was shown to be typically associated with rope-like agglomerates of SWCNTs whereas our $CNT_{SW}$ sample formed particulate bundles that had the appearance under SEM of being more like particles than fibres and were only identifiable as fibres under TEM after long sonications. Therefore, the different responses here between the lungs and the peritoneal cavity are entirely explicable on the basis of the lack of long fibres in the $CNT_{SW}$ sample.

Injection of 0wk LFC and LFA both induced an acute inflammogenic response in mice, which was anticipated given their pathogenic lengths and high aspect ratios, and known innate pathogenicities. The $CNT_{LONG1}$ samples formed diffuse agglomerates in which individual fibres could be identified. Injection of these fibres into the peritoneal cavity of mice induced all markers of inflammation to an equal or greater degree than 0wk LFA and LFC on a mass basis, with some (total protein, LDH and IL-6) persisting above the effects of vehicle only 7d post-injection, even as the response in mice injected with LFA and LFC subsided. Interestingly, the 0wk $CNT_{LONG1}$ fibres also induced more granuloma lesions 7d post-injection than 0wk LFA on a mass basis, consistent with the higher inflammatory response observed here in some of the *in vitro* assays, and also consistent with the greater pathogenicity shown previously [9]. Therefore, pathogenicity of certain fibrous high-aspect ratio CNTs has now been shown here and elsewhere in mice when injected directly into the peritoneal cavity, as well as in rats following intratracheal instillation [15]. It is also noted that dose-dependent inflammation and fibrosis have been shown in lung and lung-associated lymph nodes in rats following long-term inhalation [19], which may be more relevant to potential workplace exposures, and it has recently been confirmed that inhaled CNTs have the ability to reach subpleural tissue - although no CNTs were observed in the nearby mesothelial cells - following inhalation by mice [20]. Therefore it would seem that in their pristine state, CNTs manufactured to pathogenic lengths that can be present in the form of discrete long fibres may show equal or greater pathogenicity than some forms of asbestos when compared on a mass basis. However, it should be noted that, due to their lighter weights, a greater *number* of CNT fibres would be present per mass unit in a sample compared to the heavier asbestos fibres.

However, when samples that had been incubated for 10wk in Gambles solution were assessed for inflammogenic potential, a different picture emerged. LFA that had been incubated for 10wk induced an inflammatory response in mice that was almost identical to that induced by LFA that had been incubated for 0wk, consistent with the lack of morphological changes or fibre shortening identified by SEM and TEM. In contrast, $CNT_{LONG1}$ and the non-durable LFC both showed a greater loss of mass, a decrease in fibre length and reduced pathogenicity. Mice injected with either of these fibres that had been incubated for 10wk showed a reduced inflammatory response at 24h or 7d, and fewer granuloma lesions after 7d. Indeed, after 10wk incubation both LFC and $CNT_{LONG1}$ induced weaker responses in mice that were statistically no different from those in mice injected with vehicle alone, albeit still higher than vehicle alone. The summary of results (Table 5) suggests that pristine $CNT_{LONG1}$ samples (0wk) were capable of inducing a more substantial inflammatory and granuloma response in mice than LFA on an equal mass basis, and therefore have a high pathogenic potential, but that long-term incubation in the Gambles solution mitigated this potential. A similar pattern, although to a lesser degree, was observed for LFC. In contrast, long-term

incubation in Gambles solution did not substantially alter the pathogenicity of LFA, or lack thereof of X607 and $CNT_{SW}$. The apparent lack of toxicity of X607 at 0wk is somewhat surprising given that at this point it is also a fibre with a high aspect ratio. One possibility is that due to the much larger fibre dimensions for X607 compared to the other fibres assessed here, on a mass basis comparatively fewer fibres would have been injected and may therefore have induced only the very weak inflammatory response observed here.

The results presented here indicate that the pathogenicity of pristine $CNT_{LONG1}$ fibres reported elsewhere [9] may be consistent with the fibre pathogenicity paradigm with regard to their length, aspect ratio, and ability to induce an inflammatory response, however they may also be subject to biodegradation under the conditions used here. It could be argued that because we injected a presumed mass of 50μg/ml based on the assumption of zero loss of mass, whereas the sample had actually showed a 30% loss of mass, the loss of toxicity in the $CNT_{LONG1}$ sample could be due to injecting smaller actual doses. However, for a 30% loss of mass we saw a substantial loss of toxicity in the $CNT_{LONG1}$ 10wk compared to 0wk. In contrast, LFA showed an apparent loss of 25% mass and was also injected at a presumed mass of 50μg/ml but no loss of toxicity was observed. TEM data also indicate fibre shortening in the $CNT_{LONG1}$ samples over time which was not present for LFA; therefore we speculate that the observed loss of toxicity with incubation in Gambles solution can be attributed at least in part to the decreased proportion of longer fibres. A note should be made of the difficulty in measuring lengths of CNTs that are agglomerated and bundled as the CNT ends are not readily seen, and so the absolute number of fibres in each size range may be subject to large errors. Therefore, we stress that the average lengths and proportion of long fibres as measured by TEM should be considered as guides only, rather than absolute values. However, as the measurements were done “blind”, and repeated by a second operator in triplicate, we suggest there is a trend towards shortening of $CNT_{LONG1}$ fibres upon increased incubation which must be associated with dissolution and/or breakage.

A question remaining is exactly what kind of chemical attack caused the loss of mass and fibre shortening in the $CNT_{LONG1}$ samples that was not successful in the other CNT samples, which showed greater durability during the experimental period, with the exception of the possible slow loss of mass in the $CNT_{TANG2}$ sample over 24wk. It has been suggested that synthesis defects (such as point defects, or 5-membered or 7-membered carbon rings in the sidewalls causing strain) and/or removal of impurities during or after CNT synthesis, can introduce defects into the fibres, which could act as points of weakness for chemical attack and result in breakdown of CNTs [5]. It is beyond the scope of this study to pursue these kinds of mechanistic questions in depth, but it is interesting to speculate that something like this may have happened if our $CNT_{LONG1}$ sample had a particularly high number of surface defects. Also, if metal contaminants were dissolved during the incubation period, possibly contributing to the loss of mass, their dissolution may have left behind surface defects in the CNTs. These defects may have then been vulnerable to further attack, perhaps leading to CNT breakage, contributing to fibre shortening. Interestingly, three recent studies have shown the biodegradation of SWCNTs when incubated in the presence of oxidizing environments [21-23], two of which also pointed to an enzyme-mediated mechanism of

degradation [21, 23] and reduced inflammogenic potential of the degraded tubes [21]. The biodegradable SWCNTs carried carboxylic acid groups on their surfaces, and in one of the studies only these kinds of SWCNTs were shown to undergo biodegradation, whereas other kinds of surface functionalizations or pristine SWCNTs did not [22]. This led the authors to speculate that the SWCNT-COOH bond was intrinsically vulnerable to oxidative attack and subsequent CNT degradation. These studies did not address biodegradation in MWCNTs and we have not carried out an analysis of surface functional groups, nor is it likely that our Gambles solution was sufficiently oxidising for such a mechanism to take place, but in any case, our study, along with recent others, suggests that different CNTs may have differing vulnerabilities to biodegradation.

In conclusion, we found that of the four types of CNTs tested here, two showed virtually 100% durability after 24wk incubation in a simulated biological fluid. We observed a possible loss of ~25% mass in our $CNT_{TANG2}$ sample over 24wk incubation, but this did not reach statistical significance due to the high variation in sample recovery at 0wk, and was also not evident at any of the other time-points. $CNT_{TANG2}$ [9] and $CNT_{SW}$ (here) also showed little to no inflammogenic potential, most likely due to their tightly agglomerated states, which presumably minimised the number of discrete fibres and thus presented to the mouse as particles that could be cleared. $CNT_{SPIN}$ was not able to be assessed reliably for inflammogenic potential due to dispersal difficulties. In contrast, although pristine $CNT_{LONG1}$, chosen for this study as it contained discrete long thin fibres, has been shown here and elsewhere [9] to induce an asbestos-like response in mice, we found that long-term incubation of this sample in Gambles solution caused loss of mass, decreased the proportion of long fibres, and led to a mitigation of pathogenicity. This was an unexpected finding that suggests that durability and pathogenicity may not necessarily be consistent across all types of CNTs. However, given the substantial response induced in mice by the pristine fibres – greater (on a mass basis) than LFA, which has long been associated with the development of asbestos-related diseases - and the fact that at least two of the four types of CNTs assessed here did show complete durability, and a third was largely biodurable with the possibility of a slow loss of mass over long-term incubation, we would suggest that CNTs which are potentially of pathogenic fibre dimensions should be treated with a high level of caution in the workplace to avoid inhalation, as the majority of CNTs may be expected to show biopersistence. Clearly, though, if a CNT can be manufactured with some kind of surface defect that makes it vulnerable to chemical attack and biodegradation in biological systems, or manufactured to form clump-like agglomerates, without compromising the application for which the CNT had been designed, these may be useful ways to minimise the potential biological hazard.

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# 6. Appendix I

## 6.1 Total and differential cell counts

The total number of lavaged cells obtained 24h post-injection from mice injected with each sample type was averaged for each time-point and is shown in Figure 17. Differences in total cell counts in mice 24h post-injection with the same sample type incubated for 0wk or 10wk were assessed by unpaired t-tests. Incubation for 10wk compared to 0wk did not significantly alter the total cell count at 24h post-injection for any of the groups except LFC, where mice injected with fibres that had been incubated for 10wk yielded a significantly lower total cell count compared to mice injected with LFC fibres that had been incubated for 0wk. The total cell count was also lower in $CNT_{LONG1}$ 10wk compared to $CNT_{LONG1}$ 0wk, but this did not reach statistical significance.

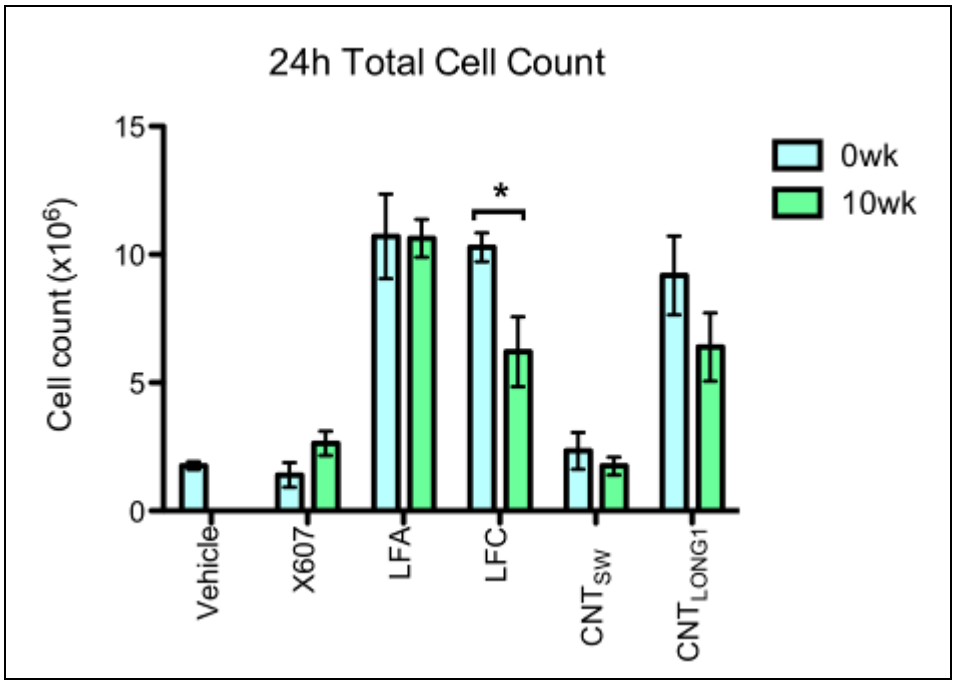


**Figure 17. Lavage total cell count in mice 24h post-injection with samples incubated in Gambles solution for 0wk or 10wk.**

The total cell counts at 24h post-injection in mice injected with LFC, $CNT_{SW}$ and $CNT_{LONG1}$ samples that had been incubated for 0wk or 10wk were compared to the cell counts in mice injected with vehicle only, as well as to counts for mice injected with incubation-matched LFA (durable fibre) and X607 (non-durable fibre). Statistical significance was assessed by one-way ANOVA with Tukey's Multiple Comparison Test (Table 6). After 0wk incubation the LFC and $CNT_{LONG1}$ samples elicited a similar total cell count in mice as those injected with the 0wk LFA, and significantly higher counts than mice injected with 0wk X607 or vehicle only. However, the cell counts in mice injected with samples of LFC and $CNT_{LONG1}$ that had been incubated in Gambles solution for 10wk were not significantly different from those in mice injected with vehicle or 10wk X607 (Note: although still higher, it is possible that statistical significance was

not reached due to the small sample numbers used in this study. This limitation of statistical significance also applies to graphs below.), and were also lower than counts in mice injected with 10wk LFA. The total cell counts in mice injected with 0wk or 10wk $CNT_{SW}$ samples were not significantly different to those in mice injected with the vehicle only or 0wk or 10wk X607, but were significantly lower than incubation-matched LFA.

**Table 6. Significance of differences in lavage total cell count in mice 24h post-injection with samples incubated for 0wk or 10wk in Gambles solution.**

| | Incubation in Gambles (wk) | Vehicle | X607 (non-durable) | LFA (durable) |
|---|---|---|---|---|
| LFC | 0 | *** | *** | ns |
| | 10 | ns | ns | *** |
| $CNT_{SW}$ | 0 | ns | ns | *** |
| | 10 | ns | ns | *** |
| $CNT_{LONG1}$ | 0 | ** | *** | ns |
| | 10 | ns | ns | ns |

(Note: in this and the following Tables, LFC has been compared alongside the CNT samples to Vehicle only, X607 and LFA to assess the difference in inflammogenic potential between the two types of asbestos tested here.)

**: $p<0.01$; ***: $p<0.001$; ns: not significant

Differences in total cell counts 7d post-injection in mice injected with the same sample incubated in Gambles solution for 0wk or 10wk were compared (Figure 18) and statistical significance was assessed by an unpaired t-test for each sample type. The 10wk incubation did not alter the total cell count at 7d in response to any of the samples except X607, where the 7d total cell count in mice treated with fibres incubated for 10wk was significantly lower compared to mice injected with fibres incubated for 0wk. The total 7d cell counts in mice injected with LFC, $CNT_{SW}$ and $CNT_{LONG1}$ samples that had been incubated for 0wk or 10wk were also compared to the cell counts in mice injected with vehicle only, or incubation-matched LFA or X607. Differences were assessed by one-way ANOVA with Tukey's Multiple Comparison Test but none of the observed differences reached statistical significance.

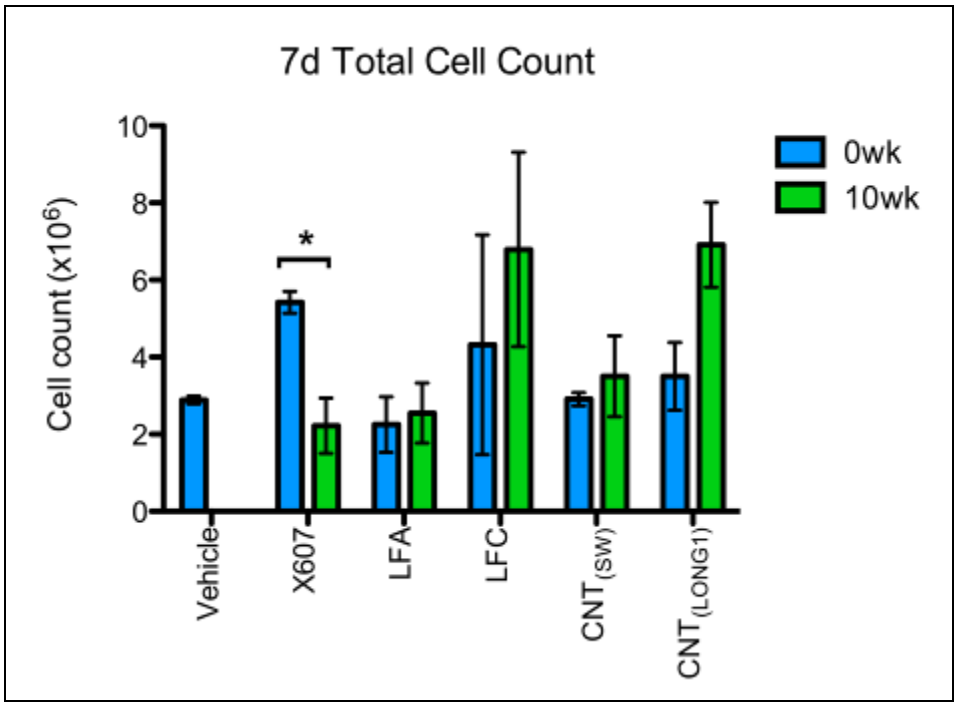


**Figure 18. Lavage total cell count in mice 7d post-injection with samples incubated in Gambles solution for 0wk or 10wk.**

A differential cell count was performed to quantify the number of PMNs and macrophages (MAC) in the peritoneal cavities of the mice for each treatment. The differential cell count at 24h post-injection is shown in Figure 19. The number of PMNs present in mice injected with the same sample type that had been incubated for 0wk or 10wk was compared using an unpaired t-test but was not found to be statistically significant for any sample.

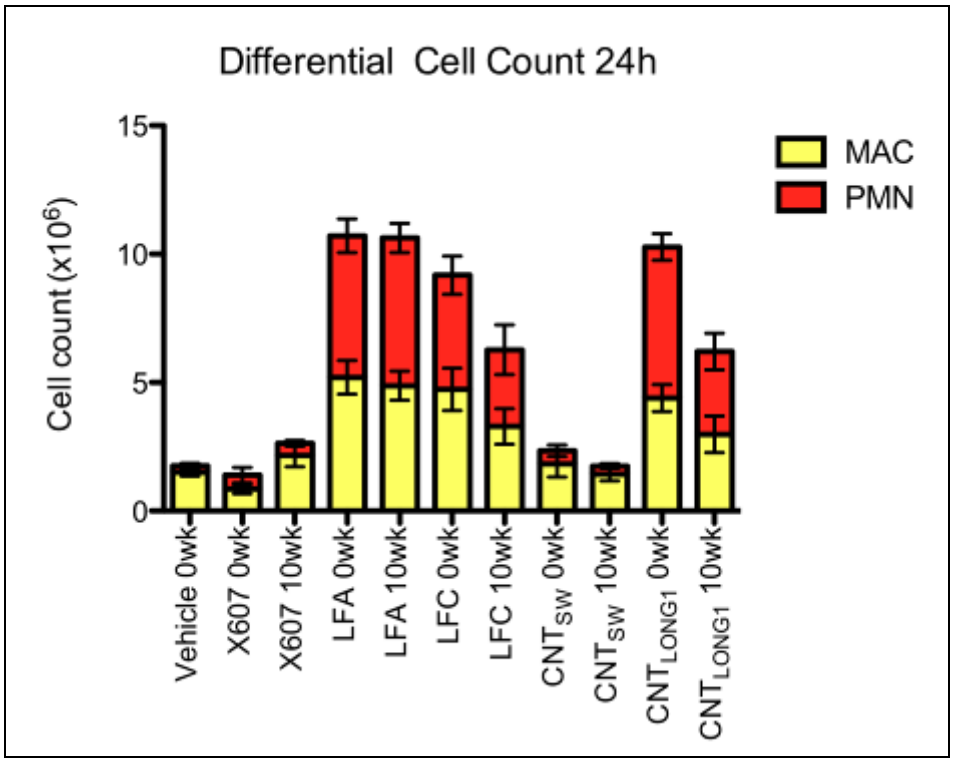


**Figure 19. Lavage differential cell count in mice 24h post-injection with samples incubated in Gambles solution for 0wk or 10wk.**

The number of PMNs 24h post-injection in mice injected with LFC, $CNT_{SW}$ and $CNT_{LONG1}$ samples that had been incubated for 0wk or 10wk were also compared to the number of PMNs in mice injected with vehicle only, or incubation-matched LFA or X607, and differences were assessed by one-way ANOVA with Tukey's Multiple Comparison post test (Table 7). Both LFC and $CNT_{LONG1}$ incubated for 0wk induced a significantly higher infiltration of PMNs into the peritoneal cavity than treatment with vehicle only or X607 incubated for 0wk, but were not significantly different from 0wk LFA. However, after incubation in Gambles solution for 10wk, the numbers of PMN present in the peritoneal cavity in mice injected with LFC or $CNT_{LONG1}$ were not significantly different (although still comparatively elevated) from mice injected with vehicle only or 10wk X607. Injection of mice with 0wk or 10wk $CNT_{SW}$ induced significantly fewer PMNs compared to mice injected with incubation-matched LFA.

**Table 7. Significance of differences in number of lavage PMNs in mice 24h post-injection with samples incubated for 0wk or 10wk in Gambles solution**

| | Incubation in Gambles (wk) | Vehicle | X607 (non-durable) | LFA (durable) |
|---|---|---|---|---|
| LFC | 0 | *** | *** | ns |
| | 10 | ns | ns | ns |
| $CNT_{SW}$ | 0 | ns | ns | *** |
| | 10 | ns | ns | *** |
| $CNT_{LONG1}$ | 0 | *** | *** | ns |
| | 10 | ns | ns | ns |

(***: $p<0.001$; ns: not significant)

The differential cell count 7d post-injection is shown in Figure 20. The number of PMNs present in mice injected with the same sample type that had been incubated for 0wk or 10wk was compared and statistical significance was assessed by an unpaired t-test, but were not significant for any.

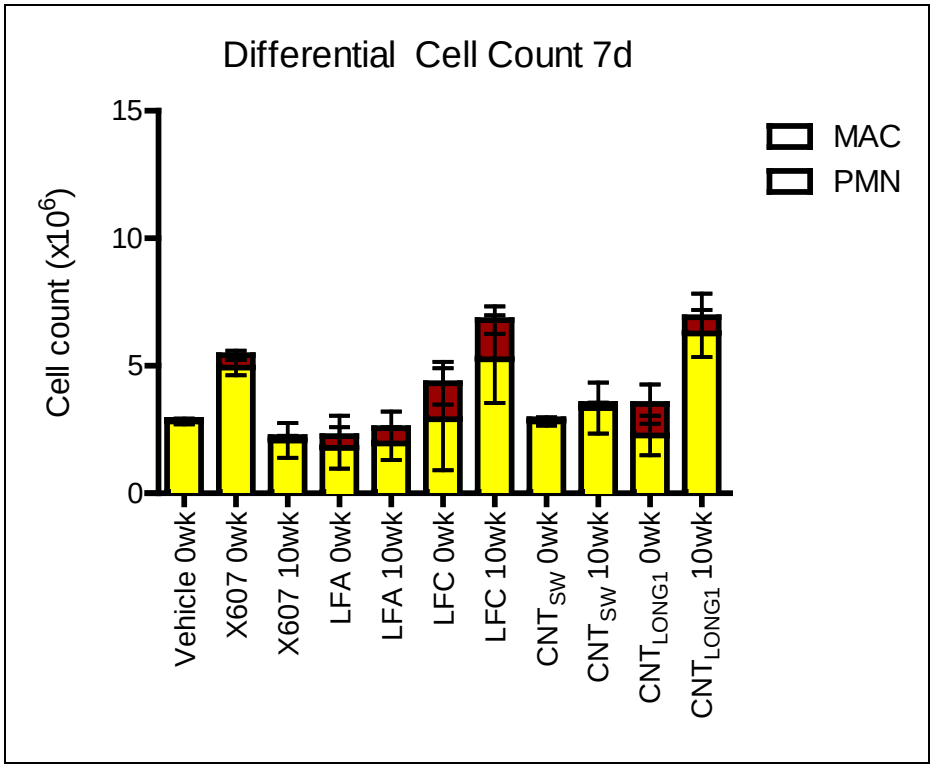


**Figure 20. Lavage differential cell count in mice 7d post-injection with samples incubated in Gambles solution for 0wk or 10wk.**

The number of PMNs 7d post-injection in mice injected with LFC, $CNT_{SW}$ and $CNT_{LONG1}$ samples that had been incubated for 0wk or 10wk were also compared to the number of PMNs in mice injected with vehicle only, or incubation-matched LFA or X607, and

differences were assessed by one-way ANOVA with Tukey's Multiple Comparison post test, but was not statistically significant for any.

The results of the total and differential cell counts suggest that treatment with $CNT_{SW}$ or X607 incubated with either 0wk or 10wk did not cause an infiltration of immune cells into the peritoneal cavity of mice 24h or 7d post-injection substantially above those in mice treated with the vehicle alone. Mice treated with LFA showed the expected response irrespective of incubation in Gambles solution, with an infiltration of immune cells in mice substantially higher than in mice injected with vehicle alone. Mice injected with CNTLONG1 or LFC that had been incubated for 10wk showed a weaker response compared to 0wk, but still higher than vehicle alone (not significant).

## 6.2 Total protein

Total protein levels in the lavage fluid of mice were determined and are shown at 24h post-injection in Figure 21. Levels present in mice injected with the same sample type that had been incubated for 0wk or 10wk were compared using an unpaired t-test but not found to be statistically significant for any.

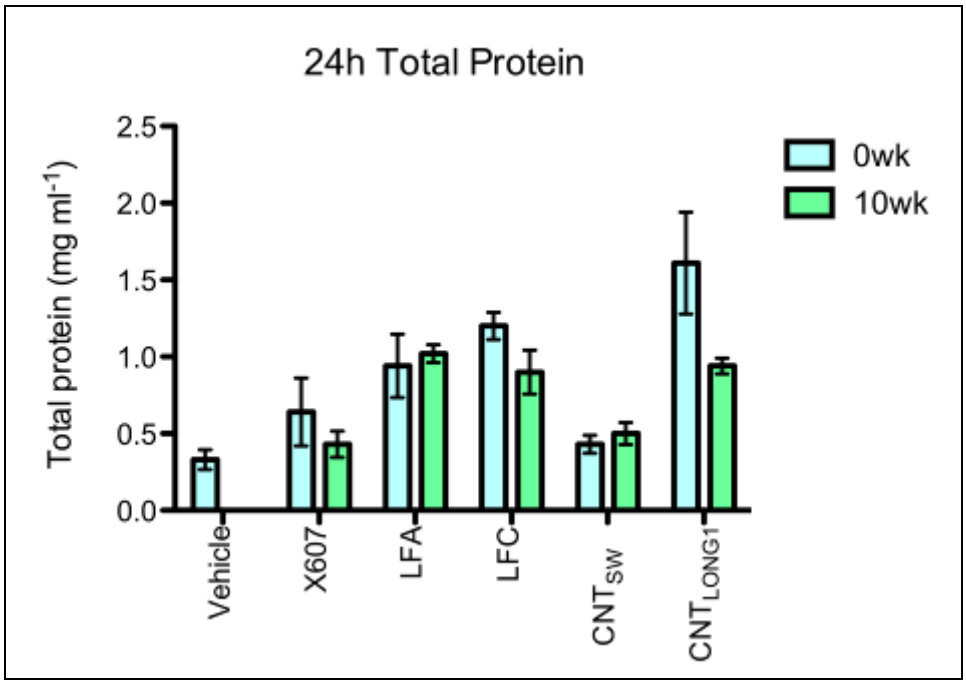


**Figure 21. Lavage total protein levels in mice 24h post-injection with samples incubated in Gambles solution for 0wk or 10wk.**

Total protein levels 24h post-injection in mice injected with LFC, $CNT_{SW}$ and $CNT_{LONG1}$ samples that had been incubated for 0wk or 10wk were also compared to levels in mice injected with vehicle only, or incubation-matched LFA or X607, and differences were assessed by one-way ANOVA with Tukey's Multiple Comparison post test (Table 8). 24h post-injection, incubation of samples in Gambles solution significantly altered the inflammogenic potential of LFC and $CNT_{LONG1}$, with high protein levels compared to mice injected with vehicle only after 0wk incubation, but these sample types incubated for 10wk in Gambles solution did not elicit a similar reaction, rather the lavage total

protein levels not significantly higher compared to those in mice injected with the vehicle alone.

**Table 8. Significance of differences in lavage total protein levels in mice 24h post-injection with samples incubated for 0wk or 10wk in Gambles solution**

| | Incubation in Gambles (wk) | Vehicle | X607 (non-durable) | LFA (durable) |
|---|---|---|---|---|
| LFC | 0 | * | Ns | ns |
| | 10 | ns | Ns | ns |
| $CNT_{SW}$ | 0 | ns | Ns | ns |
| | 10 | ns | Ns | ns |
| $CNT_{LONG1}$ | 0 | *** | ** | ns |
| | 10 | ns | Ns | ns |

(*: p<0.05; **: p<0.01; ***: p<0.001; ns: not significant)

Total protein levels in lavage fluid 7d post-injection are shown in Figure 22. Levels present in mice injected with the same sample type that had been incubated for 0wk or 10wk was compared using an unpaired t-test but was not found to be statistically significant for any.

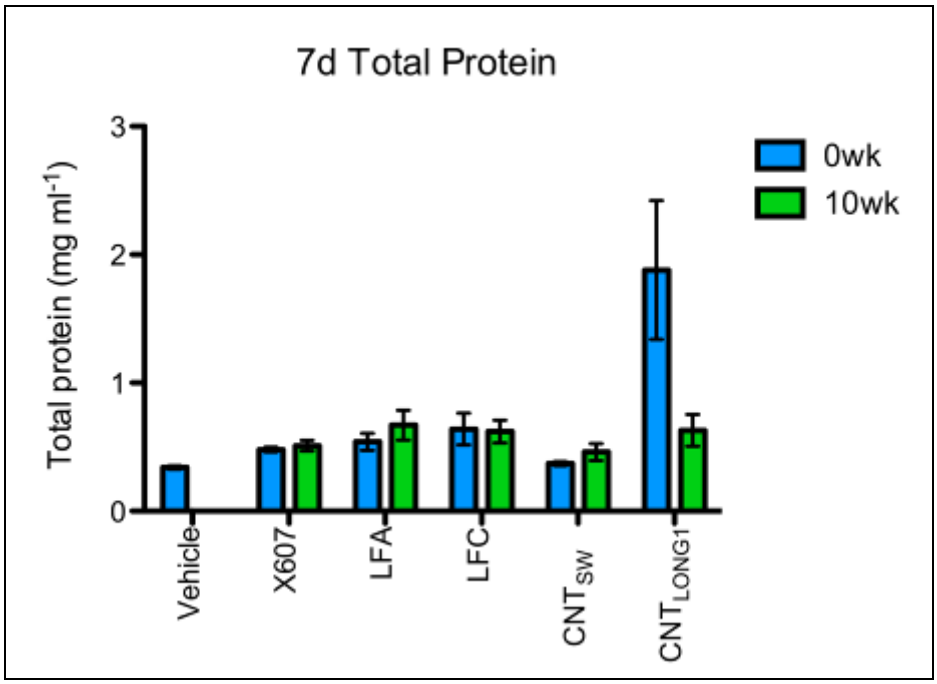


**Figure 22. Lavage total protein levels in mice 7d post-injection with samples incubated in Gambles solution for 0wk or 10wk.**

Total protein levels 7d post-injection in mice injected with LFC, $CNT_{SW}$ and $CNT_{LONG1}$ samples that had been incubated for 0wk or 10wk were also compared to levels in mice injected with vehicle only, or incubation-matched LFA or X607, and differences were assessed by one-way ANOVA with Tukey's Multiple Comparison post test (Table

9). By 7d post-injection the lavage protein levels induced in mice by all samples had diminished to levels not significantly different from mice injected with the vehicle only with the exception of $CNT_{LONG1}$ incubated for 0wk, in which mice retained significantly higher levels of total protein.

**Table 9. Significance of differences in lavage total protein levels in mice 7d post-injection with samples incubated for 0wk or 10wk in Gambles solution**

| | Incubation in Gambles (wk) | Vehicle | X607 (non-durable) | LFA (durable) |
|---|---|---|---|---|
| LFC | 0 | ns | ns | ns |
| | 10 | ns | ns | ns |
| $CNT_{SW}$ | 0 | ns | ns | ns |
| | 10 | ns | ns | ns |
| $CNT_{LONG1}$ | 0 | ** | *** | *** |
| | 10 | ns | ns | ns |

(**: p<0.01; ***: p<0.001; ns: not significant)

## 6.3 IL-6 levels

Levels of the cytokine IL-6 24h post-injection are shown in Figure 23. Levels present in mice injected with the same sample type that had been incubated for 0wk or 10wk were compared and were found to be significantly higher in mice injected with $CNT_{LONG1}$ that had been incubated for 0wk compared to mice injected with $CNT_{LONG1}$ that had been incubated for 10wk.

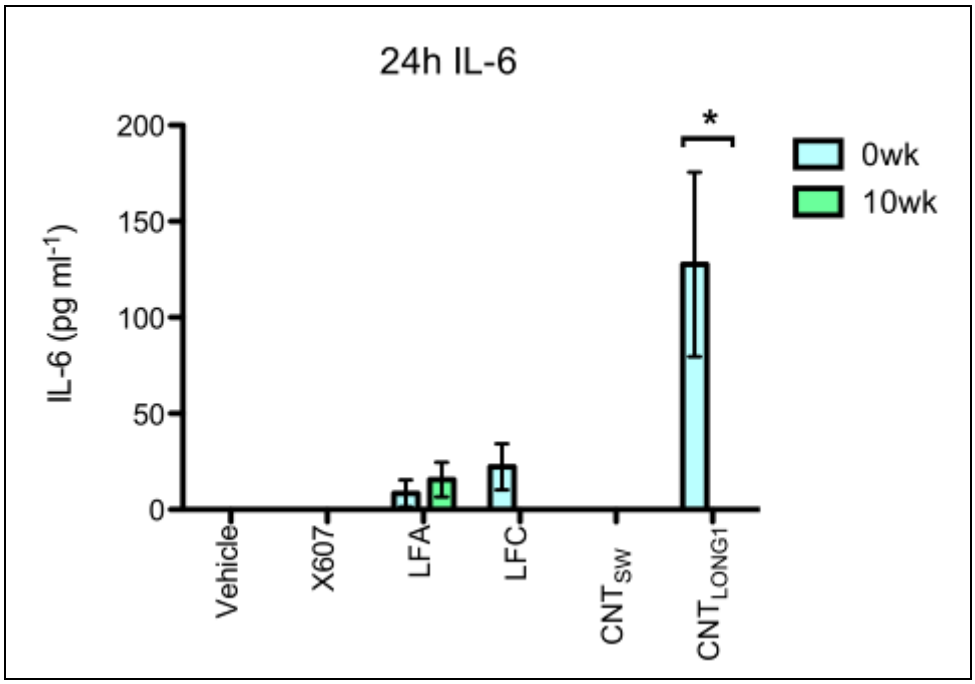

**Figure 23. Lavage IL-6 levels in mice 24h post-injection with samples incubated in Gambles solution for 0wk or 10wk.**

IL-6 levels 24h post-injection in mice injected with LFC, $CNT_{SW}$ and $CNT_{LONG1}$ samples that had been incubated for 0wk or 10wk were also compared to levels in mice injected with vehicle only, or incubation-matched LFA or X607, and differences were assessed by one-way ANOVA with Tukey's Multiple Comparison post test (Table 10). At 24h post-injection, mice that had been injected with $CNT_{LONG1}$ samples incubated for 0wk had significantly higher levels of IL-6 than mice injected with vehicle alone, or X607 or LFA incubated for the same period of time. However, $CNT_{LONG1}$ samples that had been incubated in Gambles solution for 10wk did not elicit a similar response, and levels were not significantly different from mice injected with vehicle alone.

**Table 10. Significance of differences in lavage IL-6 levels in mice 24h post-injection with samples incubated for 0wk or 10wk in Gambles solution**

| | Incubation in Gambles (wk) | Vehicle | X607 (non-durable) | LFA (durable) |
|---|---|---|---|---|
| LFC | 0 | ns | ns | ns |
| | 10 | ns | ns | ns |
| $CNT_{SW}$ | 0 | ns | ns | ns |
| | 10 | ns | ns | ns |
| $CNT_{LONG1}$ | 0 | *** | *** | *** |
| | 10 | ns | ns | ns |

(***: $p<0.001$; ns: not significant)

Levels of IL-6 7d post-injection are shown in Figure 24. Levels present in mice injected with the same sample type that had been incubated for 0wk or 10wk were compared using an unpaired t-test but were not found to be significantly different for any.

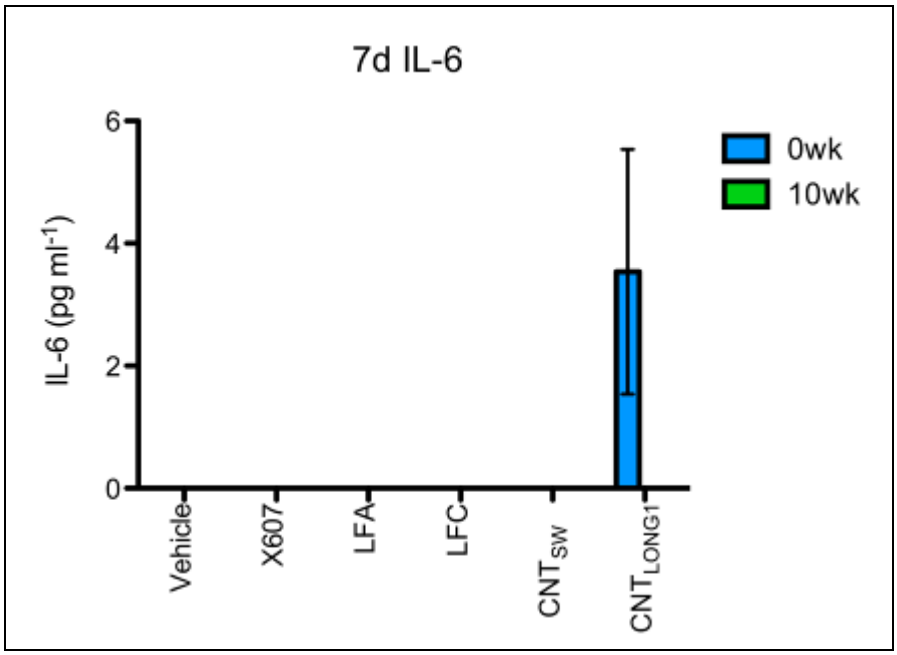


**Figure 24. Lavage IL-6 levels in mice 7d post-injection with samples incubated in Gambles solution for 0wk or 10wk.**

IL-6 levels 7d post-injection in mice injected with LFC, $CNT_{SW}$ and $CNT_{LONG1}$ samples that had been incubated for 0wk or 10wk were also compared to levels in mice injected with vehicle only, or incubation-matched LFA or X607, and differences were assessed by one-way ANOVA with Tukey's Multiple Comparison post test (Table 11). These results indicate that by 7d post-injection the levels of IL-6 had diminished from levels observed 24h post-injection, however mice that had been injected with $CNT_{LONG1}$ incubated for 0wk still had significantly higher levels of IL-6 than mice injected with vehicle alone, or X607 or LFA incubated for the same period of time. Again, $CNT_{LONG1}$ that had been incubated in Gambles solution for 10wk did not elicit a similar response, and IL-6 levels were not significantly different from those in mice injected with vehicle only.

**Table 11. Significance of differences in lavage IL-6 levels in mice 7d post-injection with samples incubated for 0wk or 10wk in Gambles solution**

| | Incubation in Gambles (wk) | Vehicle | X607 (non-durable) | LFA (durable) |
|---|---|---|---|---|
| LFC | 0 | ns | ns | ns |
| | 10 | ns | ns | ns |
| $CNT_{SW}$ | 0 | ns | ns | ns |
| | 10 | ns | ns | ns |
| $CNT_{LONG1}$ | 0 | * | * | * |
| | 10 | ns | ns | ns |

(*: $p<0.05$; ns: not significant)

Together, these results are consistent with the total and differential cells counts in showing that the ability of $CNT_{LONG1}$, and to a lesser degree LFC, to induce a cytokine response was significantly attenuated by incubation in Gambles solution.

## 6.4 LDH levels

Levels of LDH 24h post-injection are shown in Figure 25. Levels present in mice injected with the same sample type that had been incubated for 0wk or 10wk was compared using an unpaired t-test but were not found to be significantly different for any.

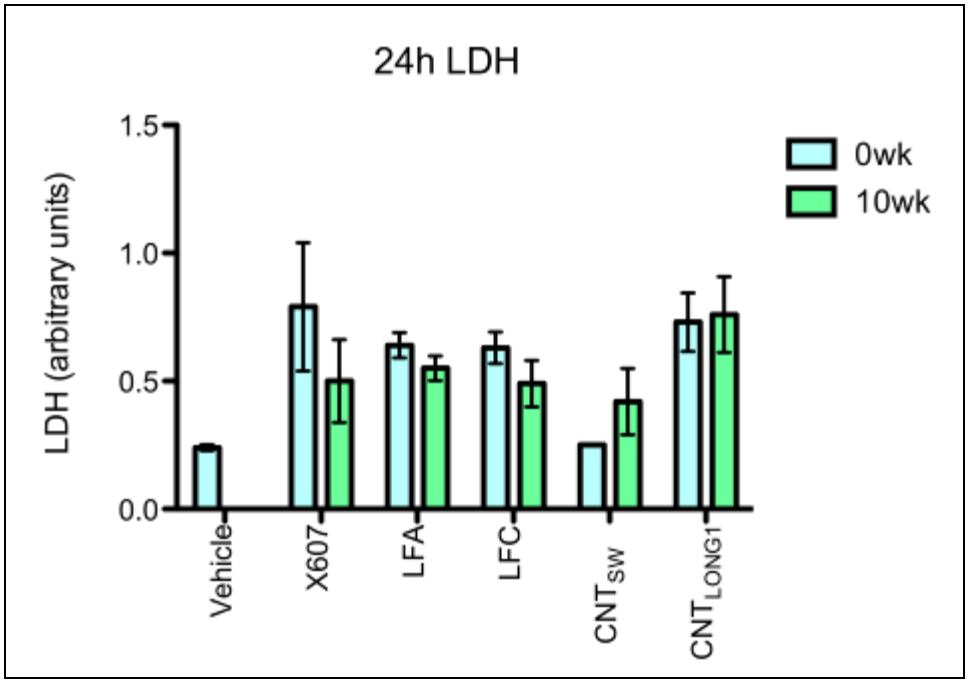


**Figure 25. Lavage LDH levels in mice 24h post-injection with samples incubated in Gambles solution for 0wk or 10wk.**

LDH levels 24h post-injection in mice injected with LFC, $CNT_{SW}$ and $CNT_{LONG1}$ samples that had been incubated for 0wk or 10wk were also compared to levels in mice injected with vehicle only, or incubation-matched LFA or X607, and differences were assessed by one-way ANOVA with Tukey's Multiple Comparison post test but were also found to be not significant for any.

Levels of LDH 7d post-injection are shown in Figure 26. Levels present in mice injected with the same sample type that had been incubated for 0wk or 10wk were compared using an unpaired t-test and found to be significantly lower in mice injected with $CNT_{LONG1}$ that had been incubated for 10wk compared to mice injected with $CNT_{LONG1}$ that had been incubated for 0wk.

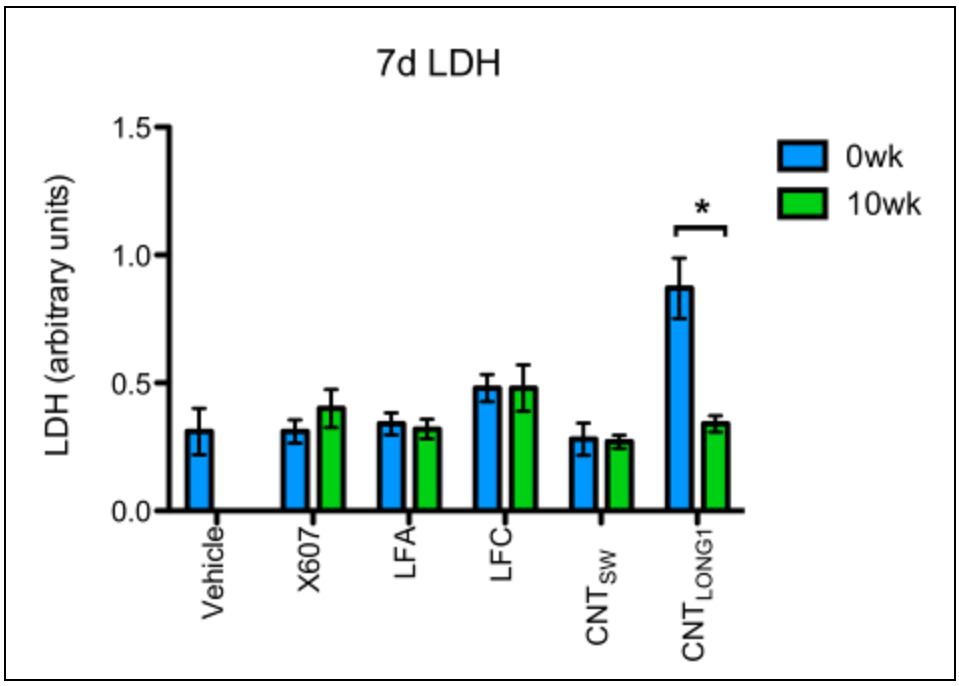


**Figure 26. Lavage LDH levels in mice 7d post-injection with samples incubated in Gambles solution for 0wk or 10wk.**

LDH levels 7d post-injection in mice injected with LFC, $CNT_{SW}$ and $CNT_{LONG1}$ samples that had been incubated for 0wk or 10wk were also compared to levels in mice injected with vehicle only, or incubation-matched LFA or X607, and differences were assessed by one-way ANOVA with Tukey's Multiple Comparison post test (Table 12). These results indicate that by 7d post-injection, mice that had been injected with $CNT_{LONG1}$ samples incubated for 0wk still had significantly higher levels of LDH than mice injected with vehicle alone, or X607 or LFA incubated for the same period of time. Levels in mice injected with $CNT_{LONG1}$ samples that had been incubated in Gambles solution for 10wk did not elicit a similar response, with lavage LDH levels not significantly different from those in mice injected with vehicle alone.

**Table 12. Significance of differences in lavage LDH levels in mice 7d post-injection with samples incubated for 0wk or 10wk in Gambles solution**

| | Incubation in Gambles (wk) | Vehicle | X607 (non-durable) | LFA (durable) |
|---|---|---|---|---|
| LFC | 0 | ns | ns | ns |
| | 10 | ns | ns | ns |
| $CNT_{SW}$ | 0 | ns | ns | ns |
| | 10 | ns | ns | ns |
| $CNT_{LONG1}$ | 0 | *** | *** | *** |
| | 10 | ns | ns | ns |

(***: $p<0.001$; ns: not significant)

## 6.5 Granuloma response

The granuloma response in mice 7d post-injection is shown in Figure 27. Lesion areas measured in the diaphragms of mice injected with the same sample type that had been incubated for 0wk or 10wk in Gambles solution were compared using unpaired t-tests and were found to be significantly lower in mice injected with $CNT_{LONG1}$ that had been incubated for 10wk compared to mice injected with $CNT_{LONG1}$ that had been incubated for 0wk.

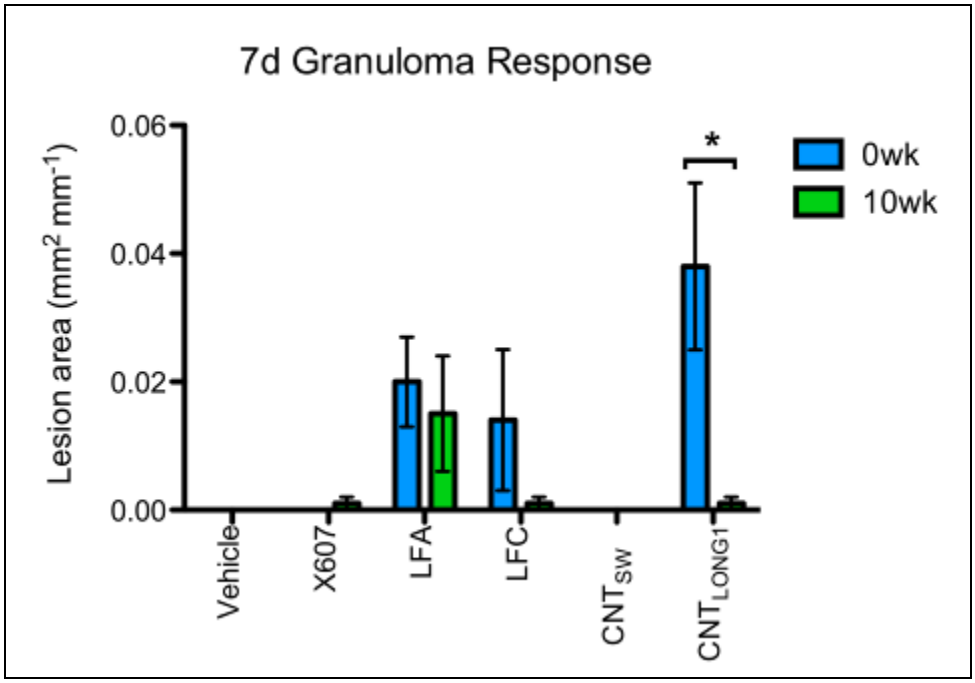


**Figure 27. Granuloma lesion area in the diaphragms of mice 7d post-injection with samples incubated in Gambles solution for 0wk or 10wk.**

LDH levels 7d post-injection in mice injected with LFC, $CNT_{SW}$ and $CNT_{LONG1}$ samples that had been incubated for 0wk or 10wk were also compared to levels in mice injected with vehicle only, or incubation-matched LFA or X607, and differences were assessed by one-way ANOVA with Tukey's Multiple Comparison post test (Table 13). These

results indicate that 7d post-injection, mice that had been injected with $CNT_{LONG1}$ samples incubated for 0wk developed significantly more granulomas than mice injected with vehicle alone, or X607 incubated for the same period of time. $CNT_{LONG1}$ samples that had been incubated in Gambles solution for 10wk did not elicit a similar response.

**Table 13. Significance of differences in granuloma lesion area in mice 7d post-injection with samples incubated for 0wk or 10wk in Gambles solution**

| | Incubation in Gambles (wk) | Vehicle | X607 (non-durable) | LFA (durable) |
|---|---|---|---|---|
| LFC | 0 | ns | ns | ns |
| | 10 | ns | ns | ns |
| $CNT_{SW}$ | 0 | ns | ns | ns |
| | 10 | ns | ns | ns |
| $CNT_{LONG1}$ | 0 | * | ** | ns |
| | 10 | ns | ns | ns |

(*: $p<0.05$; **: $p<0.01$; ns: not significan