on the cell membrane.[54,259] DDSs targeting a wide variety of diseases other than cancer have also been investigated. Some examples are described below.

As compared to alginate microspheres alone, a composite of CNTs and alginate microspheres exhibited improved drug encapsulation efficiency, resulting in decreased drug leakage. Hence, the release of theophylline, a drug used to treat respiratory diseases, was extended, suggesting a potential for application of this composite to prolong the sustained therapeutic effects of encapsulated drugs.[260] Moreover, a study showed that CNTs successfully coupled to a therapeutically active molecule could be delivered to cells of a pathogenic organism.[261−263] In addition, because of their distinct mechanism of action on resistant strains against which existing antibiotics are ineffective, CNTs have the potential to be an innovative therapy.[264] CNTs are reported to suppress bacterial proliferation.[265−268] Attempts have been made to treat diseases by immune activation or vaccination with modified CNTs. For example, a neutralizing B cell epitope conjugated to CNTs induced intensive antipeptide antibody responses to hand-foot-and-mouth disease virus, suggesting its potential as an immunotherapy.[269]

The use of CNTs in gene delivery systems is also under investigation. For example, DNA-wrapped MWCNTs prepared by sonication (because they are well and stably dispersed by sonication) are likely to have applications to gene therapy.[256] A composite consisting of MWCNTs with biomolecules immobilized by the addition of a polyamidoamine dendrimer was found to be a promising DDS for a wide variety of genes.[270] Regarding antisense therapy, two problems with antisense nucleic acids, rapid decomposition and poor diffusibility in the cell membrane, impose limitations on its application to clinical treatment. When bound to SWCNTs, however, antisense-myc was readily internalized by HL-60 cells and continued to control intracellular genes.[271] Furthermore, more than one report is available on the introduction of short interference RNA (siRNA) in cells using CNTs as a delivery system.[272−275] According to a 2010 report, the gene transfer efficiency is high at 95%, with no cytotoxicity observed. In conclusion, research aimed at the application of CNTs to gene DDSs has increased dramatically. While their application to gene therapy is expected, CNT-based gene DDSs may also be an important tool in biological research.

2.6. Other Biological Applications

In addition to the above-described applications for cancer treatment, regenerative medicine, implants, and DDSs, CNTs are expected to have biomaterial application in a wide variety of therapeutic settings.[276]

CNTs have a great potential for use as sensors and actuators in nanomedicine[89] and as sensors and stimulants in nerve tissue. Neuroblastoma NG108 and rat primary peripheral neurons produced high voltage-activated currents when electrically stimulated through conductive SWCNT films, demonstrating the electrical coupling of SWCNTs and neurons. This finding suggests that SWCNTs can be used to effectively control nerve tissue stimulation.[120] CNTs (because of their electrical properties) may also serve as muscle actuators or be directly applied to artificial muscles.[123,277] At present, it is technically impossible to use CNTs as a substitute for muscles in living organisms, and we hope that these studies will evolve into research on the application of CNTs as biomaterials. Furthermore, a DNA actuator based on encapsulated DNA-MWCNT was designed using a computer.[278]

Another potential application of CNTs is as an in vivo sensor to measure glucose concentrations in diabetic patients using near-infrared rays in vivo, bearing in mind that CNTs are capable of controlling far-infrared luminescence.[279] Hence, specific biomolecules adsorbed to CNTs and applied to in vivo sensors can be used to monitor a wide variety of diseases. Application of CNTs to nanosized devices injected into the body or medical nanorobots for in vivo implantation[99,280] is also under investigation.

As stated above, the electrical, thermal, and mechanical characteristics unique to CNTs are expected to give rise to new biomaterials that do not fall within the scope of existing concepts. Furthermore, CNTs, when brought into contact with various cells and tissues, may have unknown in vivo characteristics. Research into application of CNTs as biomaterials is expected to advance and lead to groundbreaking therapeutic approaches.

3. PRESENT STATUS OF RESEARCH INTO THE IN VIVO TOXICITY OF CNTs USED AS BIOMATERIALS

Currently available studies of the in vivo toxicity of CNTs mostly concern inhalation toxicity. Research into the toxicity of inhaled CNTs has been advancing rapidly since the publication of two articles by Takagi et al. and Poland et al. in 2008; the revelation that intraperitoneal administration of CNTs causes inflammation and carcinogenesis attracted worldwide attention.[281,282] These two studies used intraperitoneal administration as a surrogate for mesothelial tissue reactions to inhaled CNTs, bearing in mind that mesothelial tissue is present in both the thoracic and the peritoneal cavities. What was always problematic in these studies was that the CNTs were fibrous particles of similar size to asbestos particles.[283−287] It should be noted, however, that the toxicities of CNTs (very pure carbon particles) and asbestos (a mineral containing a large amount of impurities) are distinct. CNTs are highly flexible, whereas asbestos is rigid. Currently, intraperitoneal administration is often used to explore the mechanism of mesothelioma development and for other purposes,[80,288,289] and inhalation exposure or intratracheal administration is used to assess inhalation toxicity.[79,82,290−296] Recently, inhalation exposure studies have shown increasing accuracy, allowing extensive examination of gene expression in body tissues and blood after exposure.[297] Following these many studies, the Organization for Economic Co-operation and Development (OECD), the U.S. National Institute for Occupational Safety and Health (NIOSH), the National Institute of Advanced Industrial Science and Technology (AIST) in Japan, and other organizations have announced their findings.[298−302] Their reports showed that, as compared to asbestos, CNTs have much lower inhalation toxicity. The currently projected goal of toxicity assessment is to determine the threshold level of exposure triggering inflammation in the lung. In the near future, international criteria of exposure to inhaled CNTs will be established. Worldwide, the inhalation toxicity of few other substances has been investigated and discussed. In the context of production, use, and disposal of industrial products, CNTs are believed to be handleable, provided that safety measures based on the latest research findings are fully implemented, and that any available numerical criteria are met.[303] With respect to inhalation exposure, researchers and manufacturers of CNT-containing biomaterials should follow the same standards.

As stated in the section 1, the type of toxicity to the human body differs completely between the inhalation route and implantation route of exposure. Fewer studies have been conducted on the in vivo toxicity of CNTs biomaterials than on the inhalation toxicity of CNTs; however, the number of relevant reports has recently been increasing.[77,91,191,304] Unfortunately, all of the reported experiments assessing the in vivo toxicity of CNTs biomaterials lacked reference materials.[68] Notably, many published articles have suggested that the toxicity of CNTs biomaterials is extremely low.[91,191,305,306]

3.1. In Vivo Implantation Studies

This section reviews articles on implantation toxicity studies of CNTs as biomaterials. Most reports on local reactions following implantation of CNTs showed that mild inflammatory reactions occurred immediately after implant placement but disappeared early. Examples of such research include a study of subcutaneous implantation of alginate gel bound to SWCNTs,[307] a study of subcutaneous implantation of a poly(propylene fumarate) assembly bound to SWCNTs,[308] and a study of subcutaneous implantation of two MWCNTs with different lengths.[309] None of these studies found any indication of intense inflammatory reaction. In our study of subcutaneous implantation of MWCNTs in mice, mild inflammation persisted for about 1 week, resolved rapidly, and never turned into chronic inflammation. Histological profiling identified MWCNTs as phagocytosed by macrophages and remaining at the implantation site for a long period of time.[58] Studies of subcutaneously implanted CNTs by other researchers yielded similar results representing the body's characteristic reactions to CNTs.

Although subcutaneous implantation studies are a representative and convenient method of assessing the general biological compatibility of biomaterials, it is also necessary to study CNTs biomaterials actually implanted in organs.[191] We conducted a bone implantation study of MWCNTs used as scaffolds for bone regeneration and as biomaterials in contact with bone. After implanting MWCNTs in bone defects artificially made in mouse tibias, we observed normal bone repair, with incorporation of MWCNTs particles into repaired bone substrate. Electron microscopy detected physical bonding of the bone substrate hydroxyapatite in contact with CNT particles. These results show that MWCNTs possess an extremely high compatibility for bone tissue (Figure 5).[213] On the other hand, when SWCNTs and MWCNTs were implanted in rat gluteal muscle, acute inflammation developed and progressed to chronic inflammation.[76] Further investigations will be needed to elucidate CNT–muscle compatibility. A wide variety of interactions between in vivo implants of CNTs and various organs can be observed in the bodies of living organisms, making it possible to elucidate the reaction of living organisms to CNTs bound to endogenous molecules (e.g., albumin, hemosiderin). We think that a consensus has now been reached that the inflammatory reactions are mild and disappear early after subcutaneous implantation. At the next stage, other sites for clinical application of implants should be investigated in detail along with the biological reactions at each site.

3.2. In Vivo Kinetics

When applying CNTs to biomaterials, it is important to study their in vivo kinetics.[304,310,311] Specifically, it is necessary to determine whether CNTs circulate through the body via the bloodstream, whether they accumulate in particular organs, what reactions take place in the organ, and how they are excreted from the body. Of course, in vivo kinetics is of direct relevance in DDSs and imaging where localized accumulation of CNTs and distribution systemically via the bloodstream is expected. However, CNT composites used as implants do not enter the circulation, and even CNTs particles used topically hardly ever enter the bloodstream. It can also be hypothesized that small CNTs but not large CNTs enter the bloodstream to some extent.

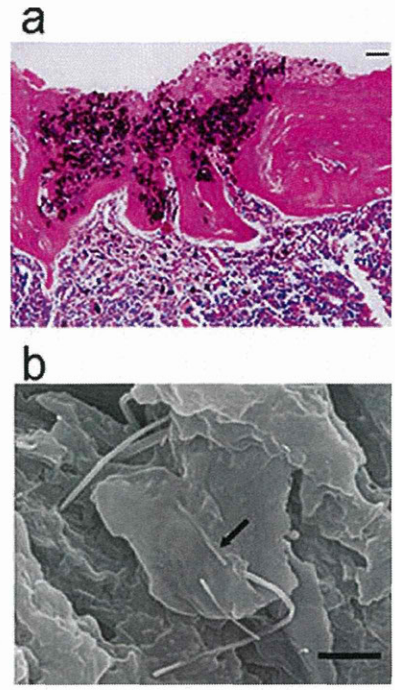

Figure 5. MWCNTs exhibiting good bone compatibility as they are absorbed in repaired bone without interfering with bone repair. (a) A histological image of a tibia extirpated 4 weeks after surgery for implant of MWCNTs in a pit drilled in tibial diaphysis after incising the anterior surface of a mouse leg. Cortical bone and a medullary cavity were normally formed to the extent of complete bone repair. The MWCNTs were found to have been absorbed in the newly formed bone tissue and enclosed in bone substrate. Hematoxylin-eosin staining. Scale bar = 100 mm. (b) An electron microscopic image of MWCNTs absorbed in repaired bone tissue at 4 weeks. The MWCNTs were found to be in direct contact with bone substrate hydroxyapatite. Scale bar = 1 mm. Reprinted with permission from ref 213. Copyright 2008 John Wiley & Sons, Inc.

The focus of in vivo kinetic studies has been on inhalation toxicity rather than on the applicability of CNTs to biomaterials. CNTs adsorbed to the lungs are thought to enter the bloodstream to some extent because the lung is the organ responsible for blood gas exchange. Therefore, it is necessary to examine the disposition of CNTs after they are inhaled and enter the pulmonary circulation. Some reports are available on the disposition of intravenously injected CNTs.[86,144,306,312–315] These studies provide valuable information on applications of CNT biomaterials and topical applications of CNTs both involving their entry and assumed entry into the bloodstream. Reported studies mostly found that CNTs entering the bloodstream are nontoxic in individuals and various organs.[191,310] For example, no sign of toxicity was

registered at least 90 days after intravenous injection of pristine SWCNTs in mice.[312] No sign of acute toxicity was registered after intravenous injection of SWCNTs or MWCNTs conjugated with diethylenetriaminepentaacetic acid (DTPA) in mice.[306] Another study verified the safety of SWCNTs 24 h after intravenous injection.[305] No toxicity was found in mice 4 weeks after receiving an intravenous injection.[310] Variable findings have been reported depending on the sites of accumulation of intravenously injected CNTs in laboratory animals. Many studies found that most CNTs were excreted in urine, with only a small amount accumulating in the liver and spleen.[87,191,316] Intravenous injection studies notably found that both MWCNTs and SWCNTs were most likely to accumulate in the liver and spleen.[310,317] Because CNTs enter capillaries and remain in various organs, it can be thought that the liver and spleen, which are rich in blood vessels, are the most likely organs of CNTs accumulation. The toxicity of CNTs accumulated in the liver and spleen is thought to be low.[86,305,306,318,319] Other organs where CNTs accumulate include the lung, urinary bladder, kidney, and gut. Although the doses used in these experiments are variable, they are often up to 20 μg/kg body weight. The solution used to disperse and inject CNTs is also variable, with phosphate buffered saline (PBS) being the most commonly used solution.[91]

Historically, various techniques for monitoring the migration of radioisotope-labeled CNTs in the body have been employed in disposition studies. ^{13}C was used in 2002, followed by ^{14}C.[320,321] In rats injected with ^{14}C-labeled MWCNTs, the liver accumulated most of the dose, followed by the lung, spleen, and kidney. The MWCNTs were gradually cleared from these organs, and quickly eliminated by excretion from the kidney. Analysis of the in vivo distribution of ^{125}iodine-labeled hydroxylated SWCNTs showed rapid distribution throughout the body and then excretion in urine and feces.[322] A study of intravenously injected SWCNTs modified with ^{111}indium-labeled DTPA and ^{99m}Tc-labeled MWCNTs found that these composites were rapidly removed from the blood via the kidney. In addition, electron microscopic examination of collected urine samples containing CNTs showed that the CNTs remained unchanged.[306,323] ^{14}C-Taurine-labeled MWCNTs were administered via the intravenous route and oral route using a stomach tube. By 10 min after intravenous administration, a large amount of ^{14}C-taurine-labeled MWCNTs had accumulated in the liver, with smaller amounts accumulating in the heart and lung; however, no accumulation was observed in any other organs. On day 90, retention of MWCNTs was found in the liver only. When administered through a stomach tube, ^{14}C-taurine-labeled MWCNTs were detected only in the stomach, small intestine, and large intestine, with no vascular migration observed. The technique for labeling CNTs and tracking their migration used in these experiments is also applicable to disposition studies following in vivo implantation.[310]

Other methods of monitoring the disposition of CNTs have been investigated. The disposition of SWCNTs (possessing intrinsic Raman spectroscopic signatures) can be monitored by Raman spectroscopy. Liu et al. quantified intravenously injected SWCNTs in the blood circulation of mice, and detected SWCNTs by Raman spectroscopy in various organs and tissues including gut, feces, kidney, and urinary bladder, and their excretion via the bile and kidney. Autopsy, histological examination, and blood biochemistry did not reveal any sign of SWCNTs toxicity in mice.[86] A real-time technique for detecting CNTs in the circulation uses photoacoustic flow cytometry.[324] Recently, echography was used to visualize CNTs and may be used in future research into the disposition of CNTs.[139,276]

The disposition of CNTs as biomaterials implanted in living organisms is a controversial issue, and some articles have suggested that SWCNTs but not MWCNTs, which have larger diameters, enter the bloodstream.[152,155] While CNTs are mostly phagocytosed by macrophages at many sites in the body, these macrophages do not return to the bloodstream; therefore, the hypothesis that macrophages do not transport CNTs into the bloodstream is convincing.[325] In 2011, CNTs were reported to migrate from subcutaneous implants to other organs and to be associated with inflammatory cytokine alterations. According to the report, CNTs did not accumulate in the liver, spleen, kidney, or heart, and although their migration to regional lymph nodes was slight, the lymph nodes remained undamaged. Inflammatory cytokine levels initially rose slightly, but then returned to their original levels. Accordingly, it was concluded that CNTs do not affect the immune system.[326] Of course, special caution should be exercised when using CNTs in particular sites, for example, the heart and lung. Their use in the ovary and uterus, which lie within the abdominal cavity, should also be avoided. In cases where CNTs are topically used at other sites, little enters the bloodstream, and if a very small amount does enter, no systemic toxicity would be expected. This is the current conclusion.

Conversely, when CNTs are used as DDSs or in imaging (where they migrate via the bloodstream), SWCNTs may be more suitable than other composites. In this case, the toxicity and accumulation of SWCNTs in nontarget organs need to be examined in detail. For this reason, the first use of CNTs biomaterials should be topical, and their systemic use should be implemented with extreme caution.

Finally, an in vitro study on the influence of intravenous CNTs on microvascular endothelial cells, which serve as a blood–tissue barrier, showed that CNTs might increase endothelial cell permeability. The reasons for increased permeability include higher levels of ROS and reconstitution of actin filaments, with possible involvement of MCP-1 and ICAM-1.[327] Further research reflecting these findings in vivo is expected.

3.3. Effects of Chemical Modifications

In the in vivo implantation studies and in vivo kinetic studies of CNTs, attention should be paid to the difference between the body's reactions to chemically modified functionalized-CNTs (f-CNTs), which can be a response to the binding partner molecule, and the body's reactions to pristine CNTs.[292,328] CNT is generally chemically modified by oxidatively destroying a C=C bond in it, attaching a carboxyl group, and reacting the carboxyl group with another molecular entity.[91,329] The main purpose of the most commonly performed chemical modification of CNTs, coupling with polyethylene glycol (PEG), is to increase their water solubility, and many studies have found that PEG alters the body's reactions to CNTs. PEG bound to CNTs was reported to stimulate immunocytes to produce inflammatory cytokines.[109,330] A study concluded that the biological toxicity of chemical modifications of PEG-CNTs is influenced by PEG. Mice injected with SWCNTs modified by both PEG and another functional group had higher neutrophil counts than mice injected with SWCNTs modified by PEG

alone.[87] In recent years, however, an increasing number of studies have shown that bound PEG reduces harmful effects.[77,331,332] A kinetic study of intravenous SWCNTs found that PEG conjugation accelerated the removal of SWCNTs from the body.[324] Numerous chemical modifications other than PEGylation can cause this phenomenon as well as a wide variety of changes in the distribution of SWCNTs in the body. For example, attachment of paclitaxel to SWCNTs resulted in increased localization in the gut and liver, and attachment of rituximab to CNTs increased levels of accumulation in the liver.[110,333] This observation is attributed to differences in the affinity for or reactivity with a wide variety of cell types in various organs depending on the molecule bound to CNTs. Size of the binding functional group and the type of chemical modification (whether covalent or non-covalent bond) can also influence the biological toxicity.[88]

Likely reasons why appropriate f-CNTs are generally safer than pristine CNTs include decreased toxicity due to the presence of functional groups of high biocompatibility and increased dispersibility in water, thus preventing their aggregation.[72,75,86,263,331,334–336] On the other hand, new forms of toxicity can emerge. In the application of particulate CNTs, f-CNTs are used in almost all cases. For this reason, it is necessary to build a library of data at least on representative f-CNTs, and, in particular, on the differences in reactions in vivo between chemically modified CNTs and pristine CNTs, which can be accessed by researchers worldwide.

3.4. Carcinogenicity Studies

Few in vivo studies have been conducted on the carcinogenicity of CNTs biomaterials implants. In the intraperitoneal administration studies to investigate inhalation-related mesothelioma carcinogenesis and its mechanism, the abdominal cavity, where mesothelial tissue is present, was used as a surrogate for the thoracic cavity.[281,282,288] Entry of intraperitoneally administered CNTs biomaterials into the abdominal cavity is unlikely. Conversely, use of CNTs in parts of the body from which entry into the abdominal cavity is likely (e.g., uterus, ovary) should be avoided. Even when CNTs biomaterials were implanted in common sites, nothing more than very mild transient acute inflammation developed, with no finding of carcinogenicity reported to date. Carbon, a substance of high biocompatibility, is very unlikely to be carcinogenic. Carcinogenesis might result, only if inflammation were persistent at the site of implantation. Because CNTs are fibrous nanoparticles, they have not been used as biomaterials. Subcutaneous implantation of CNTs has resulted in only brief, very mild inflammation. Persistent chronic inflammation is unlikely, provided that the site of implantation is appropriate.[58] However, it should be noted that the impurities and chemical modifier molecules present in CNTs can be carcinogenic.

In fact, no methodology has been established to assess the in vivo carcinogenicity of biomaterials whether they are particulate substances like CNTs or bulk biomaterials. We developed a new tool for assessing the carcinogenicity of CNTs involving subcutaneous implantation in genetically modified cancer-prone mice.[98] No carcinogenesis was detected in these mouse recipients of subcutaneous CNTs implants. This experimental study is described in detail in section 5.

3.5. Oxidative Stress

Because of its association with apoptosis and carcinogenicity, oxidative stress is a good indicator of toxicity. Whether CNTs induce oxidative stress is somewhat controversial. In vivo studies have revealed CNT-induced changes in oxidative stress markers. For example, intravenously injected SWCNTs induced high levels of oxidative stress markers in the lung and liver,[312] and a study with the antioxidant vitamin E found that SWCNTs played a major role in the induction of oxidative stress.[337] Hence, SWCNTs are likely to induce oxidative stress.[191] On the other hand, gene expression analysis in the liver and spleen found that intravenously injected MWCNTs significantly raised the level of the oxidative stress marker NAD(P)H in mice.[338] However, the prevailing opinion is that MWCNTs do not induce very much oxidative stress.[339–341] Even if oxidative stress is induced and is due to an essential property of CNTs, the underlying mechanism remains unclear. Metal catalysts remaining in CNTs have been suggested to induce oxidative stress. These facts are discussed in further detail in section 4.2.1 with a focus on cells.

3.6. Biodegradability

The biodegradability of CNTs is currently a hot research topic. Carbon fibers, which in the past were clinically used to reinforce the Achilles tendon, have been shown to fragment over a long time. This is attributable to the degradation of carbon fibers in the body.[96]

The degree of biodegradability of any biomaterial is an important toxicity issue. In the case of highly biodegradable materials, the toxicity of their decomposition products must also be assessed. On the other hand, if the material of interest is rapidly degraded in the body, the carcinogenicity and other forms of toxicity that are possibly exhibited by its original form will no longer be a concern. In 2008, pioneer investigators showed that CNTs are biodegradable.[342] Since then, the biodegradability of CNTs has been characterized as slight, and future advances in the relevant research are expected.[343–348] Even if CNTs biodegrade, however, their biodegradation occurs at extremely slow speeds; therefore, it can be thought that biodegradability has no major impact on the safety of CNTs biomaterials except in special cases such as where a single CNT fiber is used alone.

3.7. Other In Vivo Studies

In vivo studies have been conducted to assess carbon nanotube uptake and toxicity in the brain and spinal cord. A current focus is on migration of CNTs to the central nervous system (CNS), particularly to the brain.[349] Advances are expected in the application of CNTs as DDSs in the treatment of cerebral and spinal diseases. Accordingly, studies assessing neurocompatibility have been conducted using CNTs injected into the mouse brain and spinal cord.[70] However, research into CNTs interactions with the central nervous system is still at the very initial stage.[99,350]

Other studies found that CNTs caused allergic reactions,[351] and aggravated infectious disease rates.[352,353] Another study found that SWCNTs activate platelets and accelerate thrombus formation in the microcirculation.[354] These biological reactions to CNTs biomaterials are important and have to be examined extensively.

More recently, a nanoparticle-adhering protein was reported to possibly cover a part of the nanoparticle surface, reducing the targeting activity of nanoparticles in the body.[355,356] This phenomenon is called "protein corona formation" and discussed again in section 4.3.

dx.doi.org/10.1021/cr400341h | Chem. Rev. 2014, 114, 6040–6079

3.8. Body Size Differences between Humans and Small Animals

What should always be kept in mind in medical research is that results from animal experiments can differ from actual clinical findings.[357−360] Traditionally, small animals have been used in most animal experiments. It remains unknown whether assessments of CNTs toxicity shown in vivo in small animals are reproducible in humans, which have larger organs. In particular, the toxicity of small particulate substances has not been controversial and may be negligible as the body size increases. Conversely, the effects on finer structures of individual organs may increase the toxicity.

Differences in blood vessel thickness depending on animal body size can impact the disposition of CNTs. Most blood vessels are thicker in humans than in small animals. However, the thickness and structure of the terminal microvessels are thought to be nearly the same in different animal species. Hence, the migration of CNTs from tissue to the bloodstream and the obstruction of blood vessels by CNTs transported via the bloodstream are reproducible in small animals. For this reason, CNTs biomaterials can be deemed safer in humans because of the greater thickness of their central blood vessels, provided that no problems have been revealed by in vivo kinetic studies in small animals. Kinetic differences in the transport of CNTs (used in DDSs and imaging) through blood vessels and its dependence on animal body size must fully be taken into consideration.

Because cell size is the same in humans and small animals, the relationship between CNTs and cells and the effects of CNTs on cells are nearly the same. Therefore, even for basic body reactions to a small particulate substance, the results of animal experiments are considered to be highly representative.

Although these differences depending on animal body size may be resolved to some extent by conducting studies in larger animals such as dogs, it is difficult to maintain constant experimental conditions, making evaluation of a wide variety of CNTs impossible in large animals. As with ordinary biomaterials, for which International Standards Organization (ISO) and other standards are already available, it is reasonable to commence clinical application of CNTs biomaterials, provided that no problematic findings are obtained from assessments in small animals. It should always be borne in mind, however, that adverse reaction assessments can yield results inconsistent with findings from animal experiments.

4. PRESENT STATUS OF RESEARCH INTO IN VITRO TOXICITY OF CNTs FOR BIOMATERIALS

Cells cultured to test for inhalation toxicity can be used to assess the in vitro toxicity of CNTs biomaterials.[361−365] A large number of studies have examined the use of macrophages to test for inhalation toxicity. Because macrophages play an important role in the in vivo response to CNTs implants, inhalation toxicity data obtained using this type of cell are relevant to toxicity assessment of CNTs biomaterials.[155]

Unlike drugs and other chemical substances, CNTs are nanosized particles possessing unique properties; therefore, special cautions should be exercised when investigating CNTs in vitro. For example, because CNTs are essentially hydrophobic and insoluble in water, a surfactant must be used as a dispersant in culture experiments.[329] One article reported that the chemical properties of such dispersants altered the toxicity of CNTs.[366−371] In addition, CNTs may adsorb phospholipids and albumin in the culture broth, which are recognized by and interact with cells.[372−374] Furthermore, attention should be paid to possible reactions between CNTs and test reagents.[91,191] One study concluded that photometric methods were unsuitable because CNTs absorb light.[375−377] These factors affect the results of in vitro studies, making their interpretation difficult.

4.1. Cellular Uptake of CNTs

Cellular uptake of CNTs has been investigated in many types of cells by many researchers, and different studies have reported widely variable results. For example, SWCNTs have been reported to be absorbed by RAW264.7 cells in some studies and not in others.[340,364,372,378] Firme et al. studied the mechanism of CNTs passage (e.g., endocytosis/phagocytosis and nanopenetration) through the cell membranes of many types of cells.[91] Endocytosis is a form of active uptake of small extracellular particles (diameter $\leq$100 nm), and phagocytosis is another form of active uptake in which relatively large particles enter immunocytes such as neutrophils, macrophages, and dendritic cells. On the other hand, nanopenetration is a form of passive uptake; some authors have hypothesized that chemically modified or molecule-adsorbing CNTs enter cells by nanopenetration.[75,107,157,379−384]

We examined the cellular uptake of pristine CNTs, and reported that the mechanism of this uptake depended on the type of cell and choice of dispersant. We also reported that nonimmunocytes also actively absorbed CNTs mainly through endocytosis/phagocytosis (Figure 6).[385,386] Other researchers likewise denied the role of nanopenetration in cellular uptake of SWCNTs.[387] Adhesion to cell surfaces has been observed even in cells that do not absorb CNTs; it remains unknown whether the molecules that facilitate CNTs adherence to cells and those that facilitate CNTs absorption are identical. It has been reported that cell membrane proteins are involved in the cellular uptake of CNTs.[384,388] Furthermore, these membrane proteins may bind specifically to CNTs.[80,389] However, it will be necessary to investigate the influence of protein-containing dispersants on this binding between membrane proteins and CNTs.[369,371,385] A recent report suggested that exposure to electromagnetic waves promotes CNTs entry not only into the cytoplasm of cells, but also into the nucleus.[390] In conclusion, much remains to be elucidated about the cellular uptake of CNTs and its underlying mechanism.

To clarify the mechanism underlying the cellular uptake of CNTs, a wide variety of approaches have been developed. For example, light scattering analysis was used to qualitatively assess the cellular uptake of CNTs; a fluorescence detection technique was used to study the cell trafficking of CNTs; and 3-D dark-field scanning transmission electron microscopy was used to examine ultrastructural localization of CNTs in appropriately prepared target cells.[368,391−393] Successful monitoring of the cellular uptake and intracellular behavior of CNTs would clarify the reactions between CNTs and cells in more detail. The mechanism behind the cellular uptake of CNTs and their intracellular behavior not only has a bearing on the cytotoxicity of CNTs, but also on their pharmacokinetics when used in DDSs; thus, much more of this research is expected.

4.2. Mechanism Behind the Cytotoxicity of CNTs

Many studies have assessed the cytotoxicity of CNTs. Some early studies found that CNTs and asbestos have equivalent cytotoxicity in macrophages and other cells.[75,76,394] Recent studies, however, found that CNTs have low cytotoxicity.[155]

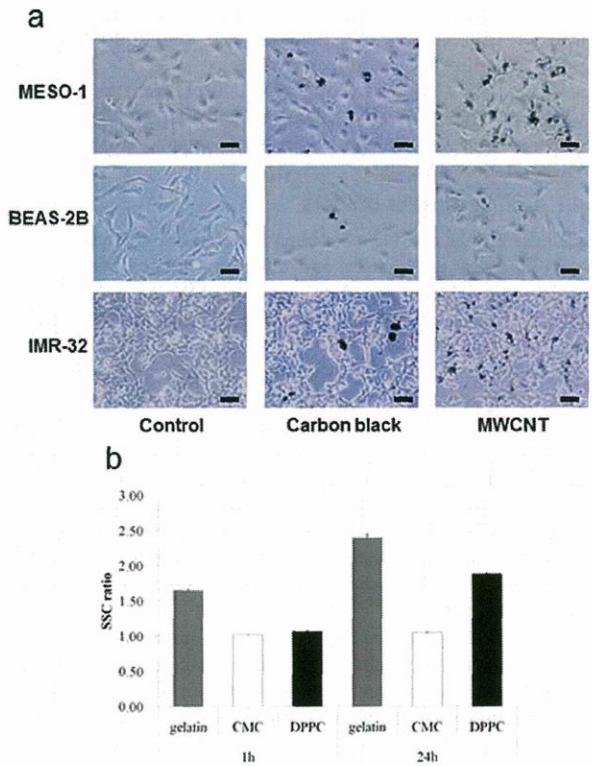

Figure 6. Cellular uptake of pristine MWCNTs varies depending on the type of cell and the choice of dispersant. (a) Combined images from bright field images and phase-contrast photomicrographs obtained 24 h after exposure of human malignant pleural mesothelioma cells (MESO-1), human bronchial epithelial cells (BEAS-2B), and human neuroblasts (IMR-32) to carbon black (CB, 50 nm diameter) and MWCNTs. Both CB and MWCNTs were absorbed in the MESO-1 cells and BEAS-2B cells, and localized around the respective exposure sites, whereas in the case of the IMR-32 cells, both CB and MWCNTs adhered but failed to be absorbed. CB and MWCNTs were added at 1 μg/mL for the treatment of BEAS-2B cells, and 10 μg/mL for the treatment of the other cells. Scale bars = 50 μm. Reprinted with permission from ref 384. Copyright 2011 Nature Publishing Group. (b) A comparison of cellular uptake in BEAS-2B observed 1 and 24 h after exposure to MWCNTs dispersed using different dispersants. Cellular uptake was determined in terms of the intensity of side scattered light (SSC) from MWCNTs absorbed in the cells using a flow cytometer. The MWCNTs dispersed in gelatin or 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were increasingly absorbed over time, whereas those dispersed in carboxymethylcellulose (CMC) were little absorbed in the cells. Reprinted with permission from ref 385. Copyright 2011 Dove Medical Press.

The reader of such in vitro cytotoxicity studies should be alert to the fact that CNTs above a certain level dose-dependently reduce cell counts regardless of cell type. This finding reflects a natural reaction of living cells to contact with foreign particulates such as CNTs. The issue is whether CNTs have a higher or lower degree of cytotoxicity than biologically safe substances.

The objective of the cytotoxicity study should also be noted. When safety is the aim of the CNTs biomaterials evaluation, concentrations in the toxic range (according to many reports; on the order of μg/mL) are used, which are much higher than the likely actual concentrations in vivo. Such high concentrations cannot occur in actual settings and can lead to an unreasonable emphasis on the toxicity. Rather, it would be more meaningful to determine the concentration at the lower limit of cytotoxicity and whether this lower limit can occur in vivo.

In addition, it should be well recognized that different types of cells can exhibit distinct responses even to the same kind of nanoparticles. This phenomenon was recently named the "cell vision" effect.[395] Exploring this effect will make it possible to clarify the mechanism for cytotoxicity. Mahmoudi et al. clarified the mechanism underlying this difference in cytotoxicity among the different cell types, and investigated the detoxification of nanoparticles.[396,397]

In all cases, when applying CNTs to biomaterials, their cytotoxicity to living organisms should be as low as possible, and by establishing the mechanism underlying their cytotoxicity, less cytotoxic CNTs can be found. A wide variety of studies to elucidate this mechanism are ongoing.[156,398,399]

4.2.1. Oxidative Stress. Oxidative stress is a focus of studies aimed at determining the mechanism underlying the toxicity of CNTs in vitro as well as in vivo. Some articles but not others have reported that CNTs may induce cytotoxic oxidative stress.[400] This cytotoxicity from oxidative stress has been attributed to the persistence of catalytic metals (Fe, Co, Ni, etc.) used in producing CNTs. Many studies have found that the cytotoxicity of CNTs increased with increase in metal content ratio.[72,368,401,402] Some CNTs contain in excess of 10% (w/w) metallic impurities, which can produce free radicals and thereby damage tissue.[263,400,403] This process can occur even after CNTs are phagocytosed by macrophage. For example, NADPH oxidase is intracellularly activated, and the resulting highly active superoxide radical kills bacteria and other pathogens. Residual Fe activates peroxides to produce hydroxyl (OH^-) radicals leading to oxidative effects on cellular proteins, lipids, and DNA. Residual Co can produce chromosome anomalies. However, a study found that Ni has no cytotoxic effects, but this finding needs to be investigated further.[75,290,404] Oxidative stress may be induced by aggregation of CNTs. Shvedova et al. found that CNTs have low in vitro cytotoxicity provided they are properly dispersed using appropriate procedures and their metallic impurities are removed.[155] Our study concluded that there was no correlation between the amount of oxidative stress from CNTs with low residual iron content and cell proliferative response or inflammatory reaction.[386,405] Carbon nanohorns, a type of carbon nanotubes without metallic impurities, were reported to be quite safe, with cytotoxicity less than 10% of the cytotoxicity of dust from road pavement.[406] However, it is unrealistic to expect that CNTs will contain absolutely no metallic impurities. Accordingly, an article discussed the limit of metallic impurity not affecting the redox properties of CNTs.[407] The susceptibility of CNTs to oxidation in the presence of metallic impurities was also analyzed.[408] In all cases, the lower was the level of metallic impurities, the lower was the level of induction of oxidative stress. Collectively, these available reports lead to the judgment that carbon purity level of 99% or more is not problematic.

On the other hand, it has long been suggested that when cells absorb CNTs, long fibers are left unabsorbed and induce oxidative stress.[281] This phenomenon is known as frustrated phagocytosis. A recent report stated that CNTs that are shorter than a given length are absorbed and not toxic, whereas longer CNTs are not absorbed but are toxic.[409−412] Consequences such as carcinogenesis may stem from prolonged inflammation

dx.doi.org/10.1021/cr400341h | *Chem. Rev.* 2014, 114, 6040−6079

due to frustrated phagocytosis in the thoracic cavity lasting long after CNTs are inhaled (Figure 7). Cytotoxicity due to frustrated phagocytosis in the context of use of CNTs as biomaterials is discussed in section 6.2.2.

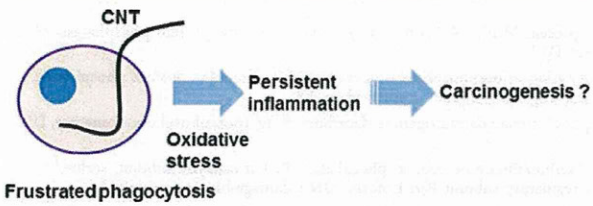

Figure 7. A schematic diagram showing a hypothesized mechanism of carcinogenesis due to frustrated phagocytosis. If left unabsorbed, long CNTs in cells can produce oxidative stress and induce inflammation. It has been suggested that a long period of persistent inflammation in the thoracic cavity following inhalation of CNTs can lead to carcinogenesis. Currently, research into the inhalation toxicity of CNTs is facing a problem with the determination of the margin of inhalation exposure that does not cause persistent inflammation.

In September 2012, the National Institute of Standards and Technology (NIST) in the U.S. reported a finding that is completely inconsistent with findings that SWCNTs protect DNA from oxidative stress.[413] Hence, no consistent conclusion has been reached concerning oxidative stress. Collectively, previous studies using many types of cells under a wide variety of conditions have led to a near consensus that CNTs do not induce oxidative stress if their aggregability and length are limited.[155] A recent study showed that chemical treatment with, for example, triethylene glycol can reduce the likelihood of aggregation in biological fluids and toxicity of even long CNTs.[414]

4.2.2. Effects on Immunity. The second issue concerns the interactions of CNTs with immunocompetent cells, including cellular uptake and subsequent intracellular transport. As such, immunocompetent cells bear a direct relationship to the safety of CNTs in vivo. Of course, pristine CNTs (because they lack antigen-presenting protein) do not cause immune reactions other than those to a foreign substance. Hence, if localized inflammation is brief, immune reactions should resolve. However, immunocompetent cells may absorb CNTs because of their nanosize, may not absorb some CNTs completely because of their fibrous form, and may orchestrate the development of an inflammatory response to residual metals and other factors in CNTs. Keeping these possibilities in mind, it is necessary to understand how immunocompetent cells respond to CNTs. Many in vitro studies have reported no response of immunocompetent cells to very pure and very short CNTs.[155,415] For example, CNTs did not have a remarkable effect on antigen-presenting cells (APCs) such as mouse macrophages (RAW 264.7 cells) and mouse bone marrow-derived dendritic cells (bmDCs).[416] An article reported that CNTs did not induce inflammatory cytokines in macrophages, whereas residual metals did.[85,401,402] If CNTs are shown to escape surveillance by immunocompetent cells, this finding will provide strong evidence for high safety of CNTs as biomaterials. Of course, it is theoretically impossible that pristine CNTs cause autoimmune disease.

4.2.3. Attempts To Lessen the Cytotoxicity. As stated above, various methods for minimizing the cytotoxicity of CNTs have been studied. For example, reducing nanotube cytotoxicity through chemical modification to change physicochemical properties and hence biological activity has been proposed. A library of 80 different surface-modified nanotubes was screened for protein bindability, cytotoxicity, and immune responses. Nanotubes had high biocompatibility, low protein adsorption properties, low cytotoxicity, and low immunostimulatory activity.[417] It has also been found that some shapes of CNTs are not cytotoxic,[309,418] and change of the graphitization temperature during CNTs synthesis alters their biological activity.[405] Hence, expectations are for the minimization of CNTs cytotoxicity. To this end and for the above-described reasons, the cellular mechanisms of CNTs recognition and the effects of the physicochemical properties of CNTs on cytotoxicity need to be clarified.[396,397,419]

4.3. CNT–Protein Interactions

CNTs used in vivo are unavoidably exposed to proteins. For this reason, successful application requires an understanding of both the adsorption of proteins to CNTs and the resulting biological responses to protein-adsorbed CNTs. While attempts to functionalize CNTs using antibodies and receptors (that are peptides or proteins) are underway,[176,420,421] the influence of proteins on pristine CNTs should be investigated. CNTs specifically adsorb fibrinogen, apolipoproteins, and albumin from blood.[422] As such, albumin is a component of most CNT dispersants in common use for toxicity experiments,[366–368,370] and it is necessary to determine whether CNTs toxicity assays actually assess pristine CNTs toxicity or albumin-adsorbed CNTs toxicity. Examination of the mode of adsorption to SWCNTs by plasma proteins fibrinogen, γ-globulin, transferrin, and bovine serum albumin using an atomic force microscope was reported, and protein binding reduced SWCNTs cytotoxicity.[423] However, the SWCNTs used in this experimental study contained many metals such as Cr, Fe, Mo, and Co, and their effect must also be taken into account.

The phenomenon in which various proteins coat the nanoparticle surface has recently been termed "protein corona" formation.[424] The protein corona is influenced by a wide variety of factors, including temperature, protein concentration, gradient concentration, protein source, and physicochemical properties of nanoparticles. The protein corona has also been reported to have major impacts on the biological reactions of cells and living organisms. For example, nanoparticles on cells and living organisms were shown to lose activity when their surface is partially covered by protein.[355,356,425–429] As such, the protein corona may determine the fate of CNTs in living organisms. In addition, changes on the nanoparticle surface caused by formation of the protein corona can alter the effects of chemically modified CNTs. Shannahan et al. compared the proteins coating MWCNTs with SWCNTs, and those coating modified with unmodified, which revealed a difference in protein composition between SWCNTs and MWCNTs and an increase in the variety of component proteins as a result of modification with COOH groups.[430] Functional deterioration of chemically modified nanoparticles has been repeatedly shown to occur; there is an urgent need to determine whether the same phenomenon can occur in CNTs.

On the other hand, to explain the decreased cytotoxicity of protein-bound CNTs, a recent study hypothesized that the human body developed a biological system mediated by protein binding to deal with exposure to numerous nanoparticles (i.e., developed a defensive mechanism against nanoparticles).[431]

Table 1. Proteins of Human Monoblastic Leukemia Cells (THP-1) Changed by Exposure to CNTs As Determined by Proteomic Analysis[a]

gene ontology term	proteins
biosynthetic process	heat shock protein β-1, elongation factor 1-δ, DNA mismatch repair protein Msh2, 6-phosphogluconate dehydrogenase decarboxylating, triosephosphate isomerase
signal transduction/cell communication	elongation factor 1-δ, DNA mismatch repair protein Msh2, 14-3-3 protein γ, serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B α isoform, protein DJ-1
carbohydrate metabolic process	6-phosphogluconate dehydrogenase decarboxylating, triosephosphate isomerase, serine/threonine-protein phosphatase PP1-α catalytic subunit, α-ketoglutarate dehydrogenase, neutral α-glucosidase AB
nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	DNA mismatch repair protein Msh2, 6-phosphogluconate dehydrogenase decarboxylating, triosephosphate isomerase, DNA damage-binding protein 1
protein metabolic process	actin related protein 2/3 complex subunit 2, serine/threonine-protein phosphatase PP1-α catalytic subunit, serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B α isoform, DNA damage-binding protein 1
catalytic process	6-phosphogluconate dehydrogenase decarboxylating, triosephosphate isomerase, α-ketoglutarate dehydrogenase, DNA damage-binding protein 1
multicellular organismal development	DNA mismatch repair protein Msh2, triosephosphate isomerase, 14-3-3 protein γ, serine/threonine-protein phosphatase PP1-α catalytic subunit
response to stress	heat shock protein β-1, DNA mismatch repair protein Msh2, DNA damage-binding protein 1, protein DJ-1
cell differentiation	heat shock protein β-1, DNA mismatch repair protein Msh2, 14-3-3 protein γ
cell cycle	DNA mismatch repair protein Msh2, serine/threonine-protein phosphatase PP1-α catalytic subunit, DNA damage-binding protein 1
transport	14-3-3 protein γ, protein DJ-1
cell death	heat shock protein β-1, DNA mismatch repair protein Msh2
organelle organization and biogenesis	actin related protein 2/3 complex subunit 2, DNA mismatch repair protein Msh2
translation	heat shock protein β-1, elongation factor 1-δ
lipid metabolic process	triosephosphate isomerase

[a]Adapted with permission from ref 463. Copyright 2011 Elsevier.

This suggests that CNTs research may elucidate the body's defensive mechanism, which is unclear.

4.4. Mutagenicity, Genotoxicity, and Apoptotic Potential of CNTs

Assessments of the mutagenicity and genotoxicity of CNTs are also important in vitro safety studies.[432−441] This is because the results from these assessments reflect the carcinogenicity of CNTs. Relatively common approaches include the Ames test, comet assay, and micronucleus test.

The Ames test, also known as the reverse mutation test, is to quantify reverse mutation (i.e., restoration of amino acid biosynthesis capability in bacteria originally deprived of that capability through mutation). Ames test studies with *Salmonella typhimurium* and other test strains have often shown that neither SWCNTs nor MWCNTs are mutagenic. A mutagenesis study showed that the frequency of mutations in mammalian cells (Chinese hamster pulmonary fibroblasts) is not altered by MWCNTs.[438,442−445]

The comet assay is a technique used to detect DNA damage in individual cells, enabling separate determination of early disorders induced at the DNA level, repair kinetics, and residual disorders. For this reason, comet assays have been performed on many types of cells exposed to SWCNTs and MWCNTs. CNTs induced DNA damage in some studies but not in others. The prevailing opinion is that any DNA damage caused by CNTs is mediated by reactive oxygen species (ROS).[446−449]

The purpose of the micronucleus test is to detect damage to the gene of interest in animal cells following administration of a test substance. Cells containing micronuclei can serve as an index of gene damage. Micronucleus test studies to assess the toxicity of SWCNTs and MWCNTs in many types of cells have yielded mixed results.[399,404,442]

Some studies of apoptosis induction by CNTs found induction of apoptosis signals in macrophages and other cells to induce apoptosis signals, while others did not find any sign of apoptosis induction.[318,378,450−452] Many cells incorporating CNTs underwent G1 phase arrest.[430] We reported that iron-rich MWCNTs caused nonapoptotic cell death.[453] On the other hand, other experiments found that highly pure MWCNTs caused apoptosis-like cell death, suggesting that the CNTs impurities have a major effect on apoptosis.[386]

In conclusion, the mutagenicity and genotoxicity of CNTs remain unclear; some studies judged CNTs to be mutagenic or genotoxic and others did not.[89,432,437,443,454−456] Results varied and depended on the cell type even within the same study.[442] In cases where genotoxicity was observed, authors hypothesized metals-induced oxidation of the DNA or suggested other hypotheses.[457] Variable results and conclusions are attributable to variable test conditions such as the dispersibility of CNTs in solution and the amount of CNTs used, as well as the amount of CNTs impurities, but not the form of CNTs (all studies assessed particulate substances). There is no current evidence in CNTs of high purity, although carcinogenicity from mutagenicity or genotoxicity calls for vigilance.[155] Further investigation will be necessary in different cell types to determine whether cells incorporating CNTs undergo apoptosis.

4.5. Cellular Signaling Events

Microarray or proteomics studies of cell signaling events induced by CNTs have been reported.[458] In a microarray study using human embryonic kidney cells exposed to SWCNTs for 2 days, decreased expression of cyclins and *cdks* (a gene affecting the G1 phase of the cell cycle) and increased expression of apoptosis-related genes were demonstrated.[318] Other researchers exposed foreskin cells to SWCNTs, and found that the expression of HMOX1, HMOX2, ERCC4, and HSPE1 and that of ATM, CCNC, DNAJB4, and GADD45A more than doubled when determined using stress and toxicity arrays and RT-PCR, respectively.[459] Using reporter gene assays of MWCNT-exposed bronchial epithelial cells, MWCNTs activated the transcription factor NF-κB to induce increased phosphorylation of p38, ERK1, and HSP27 in the MAP kinase pathway and the

dx.doi.org/10.1021/cr400341h | Chem. Rev. 2014, 114, 6040−6079

production of inflammatory cytokines.[369] Activation of NF-κB in macrophages was also reported.[460] We examined the effects of MWCNTs on cellular signaling events in osteoclasts and showed that MWCNTs suppressed osteoclast differentiation by inhibiting the nuclear migration of the transcription factor NFATc1.[217] In conclusion, the influences of CNTs on cell signaling events are important to the understanding of cellular function, and further research will be needed.

Proteomics-based studies have been conducted using keratinocytes and hepatoma cells. Results have shown changes in expression of proteins related to metabolism, stress, redox, cytoskeleton formation, apoptosis, etc., in both types of cell.[461,462] Our proteomics analysis under low-cytotoxicity conditions using monoblastic leukemia cells that do not absorb MWCNTs confirmed these changes in proteins (Table 1).[463] Such comprehensive analyses of cell signaling events increase understanding of the essential features of cellular change.[464] It is hoped that research activities will identify the pathways on which CNTs have a direct impact, and make major contributions to the assessment of the cytotoxicity of CNTs.

4.6. Choice of Cells

To date, cytotoxicity studies have often been conducted using fibroblasts and macrophages such as RAW cells. However, cellular reactions to CNTs depend on the type of cell,[396,397] and it can be thought that the reactions are specific for the organ bearing the target cells. For example, a study comparing the cytotoxicity of CNTs in the liver, spleen, and lung found that CNT-induced oxidative stress dose-dependently increased toxicity in the liver and lung, but not in the spleen.[465] We must clarify the mechanism underlying the reactions of different cell types and organs to CNTs. Because biological reactions to CNTs vary among types of cells and organs, toxicity studies using cells from likely sites of use will be needed before CNTs can be clinically applied.

For example, in a study assessing CNTs for use in nerve regeneration, human neuroblastoma cells and primary mouse neurons were exposed to MWCNTs, and their reactions were examined for effects on cell survival, oxidative stress, and apoptosis.[70] Another study examined the effects of CNTs on heart cells, specifically on impulse conduction characteristics, myofibril structure, and reactive oxygen species production in the patterned growth strands of neonatal rat ventricular cardiomyocytes. CNTs particles had much less effect than diesel exhaust particles and titanium dioxide nanoparticles.[466] To assess the use CNTs as a possible bone tissue regeneration scaffold, we examined in detail their effects on osteoblasts (bone-forming cells) and osteoclasts (bone-absorbing cells), as described in section 2.3.2.[217,218]

5. REFERENCE MATERIALS FOR SAFETY EVALUATION OF CNTs AS BIOMATERIALS

The safety of CNTs for biomaterial application remains unknown because toxicity studies have yielded inconsistent or even contradictory results as stated above. Moreover, no nanoparticle reference material has been shown to be safe to use in living organisms. All biomaterials are essentially foreign to living organisms, and hence exhibit some toxicity to living organisms. Of concern is the level of toxicity; the biological safety of CNTs cannot be assessed without conducting a toxicity study using as a reference substance that has already been recognized as safe to use in living organisms.

For example, in 2010, the cytotoxicity, genotoxicity, and apoptosis-inducing potential of MWCNTs was examined in human fibroblasts. Physiological saline admixed with a dispersant served as the only negative control. Results showed that MWCNTs exhibited dose-dependent toxicity in all dose groups as compared to the negative control, and that the cell survival rate decreased dramatically due to DNA damage, triggering pathways leading to programmed cell death. Hence, the conclusion was reached that CNTs are highly toxic. It should be noted, however, that it is scientifically incorrect to assess the toxicity of CNTs merely by comparing the results obtained in the presence and absence of CNTs. The solution (containing a dispersant) used in the reported study cannot serve as a reference for toxicity assessment. This study showed nothing more than that the experimental system used worked well, and no conclusion regarding CNTs toxicity can be drawn.

For researchers in this field, identification of an appropriate reference material for toxicity studies, which is presently unavailable, is a top priority. Kostarelos et al. pointed this out in 2009 in their review published in *Nature Nanotechnology*.[68] The reference substance must be a nanosized particulate with established biological safety. A substance can be judged as safe to use in living organisms only if it is shown to be equally or less toxic than its reference material. To render a judgment on the functioning of an experimental system, a conventional chemical substance can be used as a feasible alternative for the positive-control reference material. However, no best negative-control reference material has been found, so the safety of CNTs as biomaterials remains indeterminable.

5.1. Why Is There No Substance That Can Serve as a Reference for CNTs?

Researchers have been seeking a substance with many of the same properties as CNTs. Such references do not actually exist. Without a reference, CNTs cannot be used as biomaterials. From a broader viewpoint, any nanosized particulate substance should be considered to be a reference candidate. In fact, reference materials are specified for bulk biomaterials on the basis of this broad concept. For example, in cytotoxicity testing of bulk materials, a high-density polyethylene film serves as the negative reference material for the extraction method, and a polyurethane film containing zinc diethyldithiocarbamate (ZDEC) serves as the positive reference material. For the direct contact method, a plastic sheet for tissue culture serves as the negative reference material, and ZDEC-containing polyurethane serves as the positive reference material. These substances are specified in the ISO 10993-5 Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity (2009).[467] Hence, it is internationally accepted that a reference for a bulk biomaterial should be a bulk material of totally different nature. There is no rationale for viewing particulate materials as the only exception.

Essentially, the unfavorable criticism of nanosized fibrous particulate substances is due largely to the fact that asbestos causes cancer and other diseases. Because CNTs resemble asbestos in size and shape, their toxicity has created a stir in the media.[281,282] It should be noted, however, the inhalation toxicity of CNTs is distinct from the toxicity of CNTs biomaterials. Recently, inhaled spherical titanium oxide particles were reported to be carcinogenic;[468] however, if the judgment is made on the basis of shape and size only, no spherical nanoparticles could be used as biomaterials, and almost all nanoparticles would be inapplicable to biomaterials.

It is obvious to everyone that this claim makes no sense. Even if fibrous nature, thin and long shape, and large aspect ratio are problematic, we should keep in mind that carbon fiber biomaterials have long been used for Achilles tendon repair and other clinical purposes with absolutely no coincidence of carcinogenicity.[96,469,470] In conclusion, the most reasonable approach is to assess the toxicity of CNTs by focusing on biological reactions to nanosized particulate substances.

5.2. Biomaterials Comprising Artificial Nanosized Particles

The second reason for the inability to find a best reference material is that no nanosized particulate substance has been used as a biomaterial. This issue bears not only on CNTs, but also on a wide variety of nanoparticles, and research into biological application of nanoparticles has recently been rapidly growing. Some pharmaceuticals anchored to nanosized particles are already in clinical application. For example, abraxane, a nanoparticle substance prepared by conjugating the anticancer agent paclitaxel with albumin, degrades in the body, releasing the anticancer agent. Such conventional nanosized particles are specifically used as DDSs by making the best use of their biodegradability, and cannot be viewed in the same way as nanoparticulate biomaterials that are poorly degraded in the body.[301]

To date, only four kinds of artificial materials have been used in living organisms: chemical substances, materials with biodegradability, bulk materials lacking biodegradability, and micrometer-sized or larger particulate substances. Nanosized particulates have not been used in the body. Chemical substances, biomaterials with biodegradability, and bulk biomaterials have been used in the human body since ancient times, and many such substances have proven to be safe. For this empirical reason, researchers have been able to use these substances as references. When these substances were used as biomaterials for the first time, no scientific toxicity testing was needed. Those substances found over time to be safe to use in the human body remain in use today. Toxicity studies using some of these substances as references have been conducted to demonstrate the safety of other substances in the same category, and then using the other substances thus judged to be safe as references, the safety of still other similar substances has been demonstrated. Through this process, numerous substances have been made available for clinical application. The internationally accepted ISO standards dealing with safety evaluation are currently serving very well and have also emerged from this historical precedent.[467] The standard reference materials are known biologically safe substances rather than new reference materials evaluated to be safe for humans. Micrometer-sized or larger particulate biomaterials, for example, granular hydroxyapatite, have never posed a major problem even though they were subjected to the same safety evaluation process as conventional biomaterials.[471−473] Because CNTs and other nanosized particulate substances fall into a different category of biomaterials than micrometer-sized or larger particulate substances, the use of conventional bulk biomaterials and hydroxyapatite particles as reference materials for them is controversial. Because nanosized particulate substances have not been used in the human body, there is no implicit reference with established safety.[474]

For these reasons, obtaining a reference with confirmed biosafety in the human body for use in toxicity studies of CNTs appears to be impossible. From a broader perspective, however, otherwise unknown nanoparticles may be discovered. We considered that highly pure carbon black could serve as a reference for CNTs, because it is the primary component of the black ink used in tattoos, and also because black tattoo inks have long been injected into human bodies and are currently used by a tremendous number of people worldwide. Evidence showing that black tattoo inks are composed of nanosized carbon black particles is described below, with an overview of the biological safety of CNTs using carbon black as a reference.

5.3. Safety Evaluation of CNTs Using Nanosized Carbon Black Particles as a Reference

5.3.1. Nanosized Carbon Black Particles in Tattoo Ink. Two commercially available black tattoo inks (Sumi-Black, Unique Tattoos, Subiaco, Australia; Lining-Black, Classic Ink, Victoria, Australia) were purchased and extensively analyzed for components. Each was dried, and the resulting solid product was morphologically examined by scanning electron microscopy (SEM); particles with a nearly uniform diameter of several tens of nanometers were found to have accumulated (Figure 8a). After SEM examination, the particles were subjected to an elemental analysis using energy dispersive X-ray spectroscopy (EDS). Results showed that both inks had a C content of about 99.5 wt % and different impurity profiles, with trace amounts of Na and S detected and attributable to the surfactant added. A Raman analysis using common industrial carbon black (Vulcan XC 72, Cabot, Boston, MA) as a control revealed that Raman shift of both black tattoo inks was nearly the same as that of the control (Figure 8b). Furthermore, transmission electron microscopy (TEM) revealed that the particles in black tattoo inks had nearly the same shape as those of ordinary carbon black (Figure 8c). These findings identified the particles in tattoo inks as pure carbon black (i.e., nanosized carbon particles) as with MWCNTs.[97]

In 2012, on the other hand, a report titled "Chemical Substances in Tattoo Ink" was released from Denmark.[475] Concerning a research project implemented by the Danish Technological Institute in cooperation with Bispebjerg Hospital and the National Food Institute, Technical University of Denmark, the report explicitly described carbon black as the principal component of black tattoo ink, and toxicity assessments of carbon black found no biological safety problem.

An extremely large number of humans have received black tattoos since ancient times, and this practice has caused no major problems; tattoos are popular even today. Hence, carbon black can be described as a biomaterial that has been proven by historical evidence to be safe for use in the human body. As such, the nanosized carbon particles used in black tattoos, as with CNTs, are very pure carbon black; thus, carbon black should be considered as a good reference material for CNTs.

5.3.2. Comparison of Characteristics of CNTs and Carbon Black. To use the biologically safe carbon black tattoo ink as a reference material for CNTs, both substances should share some characteristics. Despite their considerably different characteristics, current reference materials for bulk materials have been used as international standards, and safety assessments have been conducted with no major problems. This has become feasible because of the large amount of data compiled throughout the long history of biomaterials research. However, references for nanoparticle biomaterials remain to be found. The accuracy of safety evaluation will be increased by using substances with similar characteristics in the beginning.

The characteristics (including composition, size, shape, and surface chemistry of the reference material used for CNTs [i.e.,