CHEMICAL REVIEWS

Review

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Safe Clinical Use of Carbon Nanotubes as Innovative Biomaterials

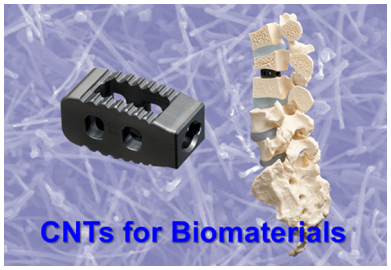
Naoto Saito,*[,†] Hisao Haniu,[‡] Yuki Usui,[‡,§] Kaoru Aoki,[‡] Kazuo Hara,[‡] Seiji Takanashi,[‡] Masayuki Shimizu,[‡] Nobuyo Narita,[‡] Masanori Okamoto,[‡] Shinsuke Kobayashi,[‡] Hiroki Nomura,[‡] Hiroyuki Kato,[‡] Naoyuki Nishimura,[⊥] Seiichi Taruta,[∥] and Morinobu Endo[§,∥]

[†]Institute for Biomedical Sciences, Shinshu University, Asahi 3-1-1, Matsumoto 390-8621, Japan

[‡]Department of Orthopaedic Surgery, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto 390-8621, Japan

[§]Research Center for Exotic Nanocarbons, and [∥]Faculty of Engineering, Shinshu University, Wakasato 4-17-1, Nagano 380-8553, Japan

[⊥]R&D Center, Nakashima Medical Co. Ltd., Haga 5322, Kita-ku, Okayama 701-1221, Japan



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1. INTRODUCTION

Carbon nanotubes (CNTs) are structurally described as sheets of six-membered carbon atom rings (i.e., graphene) rolled up into cylinders. CNTs with only one layer are known as single-walled CNTs (SWCNTs), and those with two or more layers are known as multiwalled CNTs (MWCNTs). Cup-stacked carbon nanotubes and carbon nanohorns are also sometimes called CNTs.[1−3] Currently, these very attractive carbon materials and nanomaterials are a subject of vigorous product development in a broad range of fields.[4−11] The reasons are that CNTs have useful electrical, thermal, and mechanical characteristics, and their base material performance can be improved by combination with other materials.[12−23] A recent industrial application of CNTs as an electrode additive to lithium-ion batteries is based on their excellent electrical characteristics. Addition of CNTs prevents battery deterioration and substantially lengthens time to recharging. It is doubtless that the demand for high-performance batteries will grow increasingly with multifunctionalization of personal computers and mobile phones, development of new mobile terminals, spread of electric vehicles, and other factors.[24−30] Composite materials with the excellent mechanical characteristics of CNTs have already been used in sporting goods such as golf clubs, tennis rackets, and bicycles. CNTs are also expected to have applications that reduce the weight of aircraft and automobiles.[10,14,31−35] A wide variety of advantages are gained from the use CNTs in precision parts as well. CNTs are also used in transistors and memory devices, and enhance their efficiency. The use of CNTs in various displays and TV screens continues to increase in rate. CNTs are also widely used in products designed to prevent static electricity, to shield electromagnetic waves, to store electricity, and for other purposes.[36−45] Furthermore, Japan is now facing nuclear energy issues stemming from the accident at Tokyo Electric Power Company's Fukushima No. 1 nuclear power plant. As a result, CNTs are expected to play a major role in developing new energy sources such as solar photovoltaic power generation and wind power generation.[46−52]

In the medical field, extensive research activities are underway to develop new CNTs biomaterials for use in the treatment and diagnosis of disease. For example, application of CNTs to cancer treatment and diagnosis, such as in drug delivery systems (DDSs) for treatment of cancer, hyperthermia, and in vivo imaging, has been investigated.[53−57] In a study that aimed at applying CNTs to regenerative medicine, CNTs were found to work excellently as scaffold materials for nerve and bone tissue regeneration.[58−63] Furthermore, R&D activities are underway to improve the mechanical strength and durability of implants by combining CNTs with existing biomaterials.[64−67] Besides, numerous ideas have been put forth about how CNTs can be used in the treatment of a variety of diseases.

Figure 1 shows the trend in the number of articles found in the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) (accessed 20 March 2014) by searches using "carbon nanotubes" and "biomaterials" as keywords. The number has been soaring since 2005, suggesting that CNTs research has become a highly competitive field worldwide over the past few years. Of course, numerous articles on the biological applications of CNTs do exist that cannot be captured with these two simple keywords, and the graphic representation of this trend is no more than an indicator of the increase in this research over time.

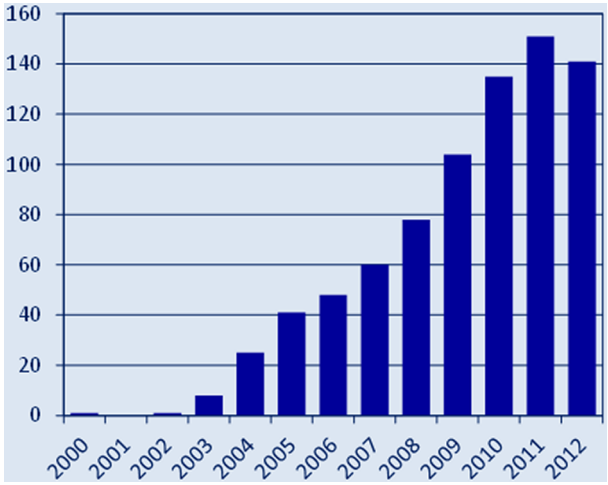

Figure 1. Time trends for the number of articles found in the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) (accessed 20 March 2014) by search using "carbon nanotubes" and "biomaterials" as keywords. Recent years have seen a rapidly increasing number of research articles on the application of CNTs to biomaterials; the number has been soaring since 2005, suggesting that the application of CNTs to biomaterials has become a highly competitive research field worldwide over the past few years. This graph indicates only a time course, and numerous articles on biological applications of CNTs do exist that cannot be captured with these two keywords.

One reason for the intense competition to find biomaterial applications of CNTs and for the great potential of CNTs to advance medical care is their small size (nanometers in diameter and micrometers in length), which makes them suitable to react with living organisms.[68−70] Hence, the size of CNTs, which is at the cell organelle level, is likely to facilitate their effect on living cells. Specifically, CNTs are similar in thickness and length to microtubules, which make up the cytoskeleton and mediate a wide variety of cellular activities such as motor protein activity.[71] Biomaterials containing CNTs of such size make reactions with cells more controllable and make treatment and diagnosis that focus on target cells more feasible, accurate, and less invasive to living organisms than conventional approaches. The second benefit from biologically applying CNTs is the ease with which they bind to a broad range of molecules thanks to the extremely high reactivity of CNT surfaces.[72,73] CNTs serve as a platform for binding multiple molecular entities such as drugs for therapeutic purposes, marker molecules, cell-binding molecules, and molecules facilitating the transfer of drugs to target tissues. Thus, CNTs can facilitate the diagnosis and treatment of

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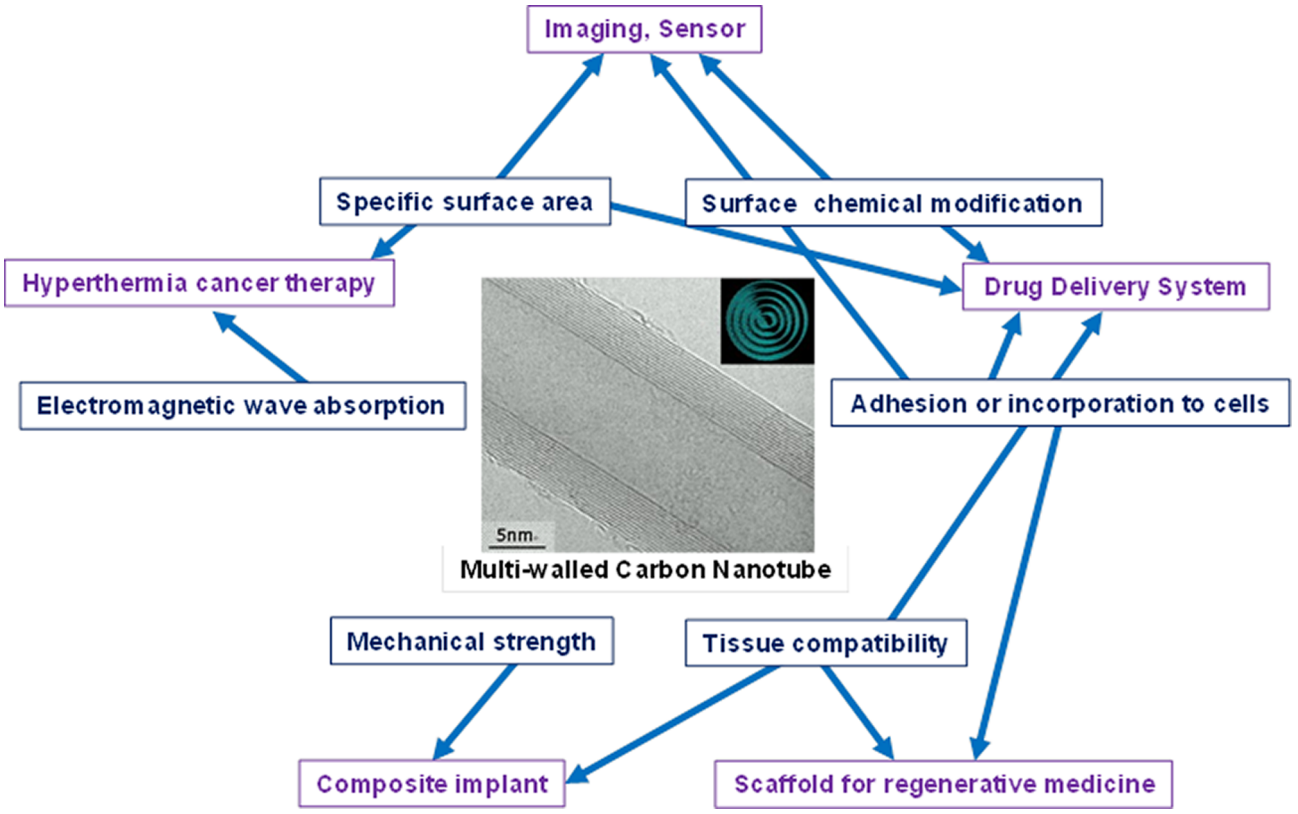

Figure 2. Biological applications of CNTs encompass a broad range of fields, many of which, in addition, represent themes of top priority in today's clinical medicine, such as cancer treatment and regenerative medicine. Modified from ref 84, which is published under the Creative Commons Attribution License.

diseases by facilitating substance recognition, adhesion, and affection to target cells. This potential is expected to lead to a groundbreaking new technology with applications to cancer treatment and regenerative medicine. In the near future, it is more likely that CNTs will be used as biomaterials for treatment and diagnosis of various diseases than for industrial purposes such as in batteries and aircraft. CNTs are of paramount importance to future advances in medical care. On the other hand, the small size and high surface reactivity of CNTs, properties that underlie their advantage as biomaterials, can adversely affect the human body. CNTs have not yet been used clinically (despite the dramatically increasing amount of research into biomaterial applications worldwide) because of safety concerns associated with implantation of CNTs devices in the body.[74−77] Currently, the safety of CNTs (primarily the safety of inhaled CNTs) is being investigated throughout the world.[78−83] Inhalation is the most likely route of external exposure of the human body to CNTs used in industrial products, so that inhalation toxicity must be determined first. It should be noted, however, that the safety profile of CNTs as biomaterials differs completely from that of inhaled CNTs.[68,69,84] Part of the safety evaluation of CNTs for biomaterial application, unlike that for inhalation, must include studies of the biological toxicity of implants in vivo. In many cases, biomaterial-specific studies must include implant toxicity, cytotoxicity, carcinogenicity, and genotoxicity studies. The safety of CNTs must be confirmed in these toxicity studies before they can be used in biomaterials. For this reason, the number of reports on the safety of biomaterials containing CNTs has been increasing.[54,68,85−89] Although these reports have demonstrated the safety of these biomaterials, researchers are still unable to reach a definitive conclusion. This is because CNTs are essentially nanoparticles, and biomaterials containing CNTs do not fall within the scope of biomaterials as traditionally conceptualized.[68,90] Of course, CNTs are not a drug, any other chemical substance, bulk material (as used herein, the term bulk material/biomaterial refers to a nonparticulate bulky material/biomaterial), or biodegradable material currently in use. Nanosized particulate substances lacking high biodegradability have not been used in the medical care field so far. Because of the nanosize of CNTs, many toxicity factors associated with nanosize will need to be investigated. Factors likely to impact the toxicity of CNTs and living organisms include thickness, length, specific surface area, and surface chemistry, as well as types of chemical modifications, defects in CNTs, and catalyst left unconsumed in the manufacturing process.[72] Factors affecting the administration of CNTs to living organisms include choice of dispersant, dispersant concentration, method of in vivo exposure, and duration of in vivo exposure. Furthermore, organ specificity, cell specificity, types and incidences of biologically adverse events, in vivo distribution, and other factors must be examined.[91] Collectively, these facts seem to suggest that developing biomaterial applications of CNTs will be difficult. Thus, absolutely no clinical applications have been found to date despite the rapid increase in the number of research articles dealing with CNT biomaterials.[10,77] However, inasmuch as applying CNTs biomaterials has potentially great benefits, the research must continue. Now is the time to review the present status based on available safety evaluation studies, to identify and resolve issues, and to implement clinical applications. Essentially, the human body consists principally of

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water and organic molecules, so life can be described as being supported by carbon.[92] To date, no problems have been reported from the use of materials consisting of ultrapure carbon, such as pyrolytic carbon used in artificial heart valves, carbon fibers used in Achilles tendon sutures, and the amorphous diamonds used in artificial finger joints.[93−96]

When reviewing, in detail, research articles by a great many researchers, it is evident that the major problem with the development of biomaterial applications of CNTs has been the lack of a particulate substance to serve as a biological safety reference material, and hence the inability to establish criteria for evaluating biological safety. This critical issue was first pointed out in 2009 by Auffan et al. in *Nature Nanotechnology*,[68] and no appropriate reference has since been found. We consider that nanosized highly pure carbon black particles are suitable as a reference material for safety evaluation of CNTs.[97,98] This is because no safety issues have appeared in the vast number of people who have black tattoos, containing principally nanosized highly pure carbon black. The use of carbon black as a reference is described in detail in section 5. Provided that same reference is used to conduct multifaceted extensive toxicity studies and provided that international standards of safety evaluation are established, it will be possible to apply CNT biomaterials in a wide range of clinical settings in the near future.

This Review covers many recent studies on biomaterial applications of CNTs mainly published between 2005 and 2013, and gives an outline of our published studies with new references. First, the findings in these studies are comprehensively discussed to evaluate the safety of CNTs as biomaterials. The way to realize safe clinical application of CNT-based biomaterials in the future is then proposed clearly. The challenge must always be kept in mind. Making the best use of all talents and abilities of researchers worldwide, this research will lead to a major revolution in the medical care field and benefit patients greatly. In this Review, we submit a proposal of paramount importance that we think will be the key to accomplishing this significant goal.

2. PRESENT STATUS OF RESEARCH INTO THE APPLICATION OF CNTs AS BIOMATERIALS

As is evident from the recent increase in the number of relevant articles, research into application of CNTs as biomaterials is advancing rapidly (Figure 1). CNTs have applications to a broad range of fields, many of which, in addition, have top priorities in clinical medicine today (Figure 2).[84,99−101] This section divides these applications into five categories: cancer treatment, regenerative medicine, implants, DDSs for noncancer targets, and other applications. Notably, many technologies utilizing CNTs are applicable to more than one of these fields. For example, the technology using CNTs as anticancer agent delivery systems is also useful for drug delivery systems targeting noncancer diseases. The technology for combining CNTs with other biomaterials is the key to successful application in new highly functional implants and in scaffolds used in regenerative medicine. Hence, this classification system was chosen only because it facilitates organization of the various published reports. In the future, classifying the studies on CNTs biomaterials with a focus on important technologies for their biological applications would be even more useful and expected to accelerate advances in relevant research.

All studies of biological applications reviewed below highlight at least one benefit of CNTs biomaterials, so these benefits are described below. The importance of these benefits has stimulated the rapid emergence and evolution of much research.[69]

2.1. Benefits from Application of CNTs as Biomaterials

The first benefit comes from the small size of CNTs. Although this benefit may have a negative impact on safety, it by far outweighs the possible risk. The following six capabilities can be attributed to the small size of CNTs:

(1) Reacting with cells by entering the cells or adhering to cell surfaces
(2) Acting on biological macromolecules and cell organelles of similar size
(3) Acting on parts of the body with fine structures
(4) Distributed via the bloodstream after intravenous injection and the like; thus they may be used in targeted drug delivery systems and in vivo imaging
(5) Rapidly eliminated from the body
(6) Having effects when combined with other biomaterials, for example, on fine structures to increase their mechanical strength

Because capabilities (4) and (5) assume that CNTs circulate in the bloodstream, the possibility that the risk of accumulation in particular organs and leading undesirable reactions to the organ outweighs the benefits must be taken into account. It is necessary to make the best use of these advantages, while minimizing the disadvantages. This is also true for other nanobiomaterials currently under investigation. Interactions between nanosized substances and living organisms will be further elucidated in the future. Nanobiomaterials are going to occupy an important position in nanomedicine, a research field that has only recently been established.[102−105] The second benefit is the ease of chemical modification. CNTs, because of their macromolecular size, have high chemical reactivity.[106] It is likely that the CNTs used in biological applications will be functionalized-CNTs (f-CNTs). When used as particles, rather than as a composite material, CNTs are likely to be f-CNTs.[73] CNTs can serve as a platform for concurrent binding of drugs, peptides, high molecular polymers, and other molecules that otherwise cannot be bound to each other (Figure 3).[107−112] Thus, it would be possible to construct CNTs with multiple functions that have not traditionally been co-occurrent, such as drug transport, cell adhesion, biomembrane transport, and release at targeted sites. For example, CNTs coupled with an anticancer agent and monoclonal antibody can be used to target cancer cells.[113,114]

There are two types of interactions with CNT surfaces: those based on covalent bonds and those based on noncovalent bonds. Of course, covalently bound substances (in contrast to noncovalently bound substances) are unlikely to dissociate from CNTs, so the appropriate method of binding must be chosen according to the target site and intended use. CNTs synthesized using the chemical vapor deposition (CVD) technique have open ends to which chemical modifiers can be bound specifically.[24] More interestingly, it is possible to transport molecules, atoms, etc., that have been inserted into the cylindrical hollow structure unique to CNTs. CNTs with such chemical modifications are called peapods because of their shape.[115,116] As such, CNT peapods can transport drugs in encapsulated form, and are expected to be increasingly

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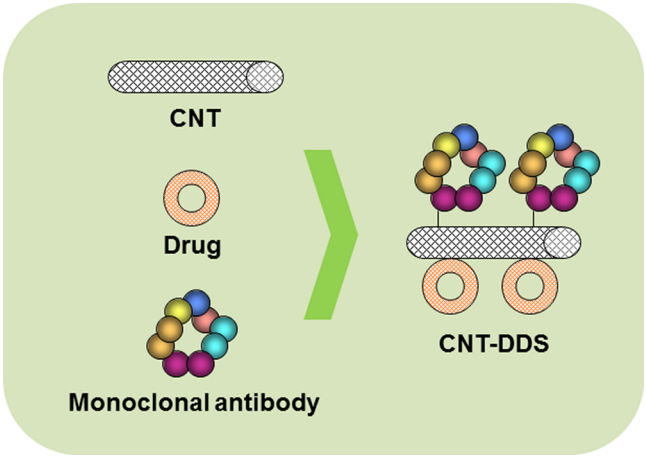

Figure 3. CNTs are capable of working as a platform for concurrently binding drugs such as anticancer agents, proteins, and peptides such as monoclonal antibodies, high molecular polymers, and other molecules that otherwise cannot be bound to each other. Making the best use of this feature, it would be possible to concurrently add to CNTs multiple functions that have traditionally been unable to concur, such as drug transportation, biomembrane passage, and release at targeted sites.

investigated because of their potential application as DDSs and in vivo imaging.[117−119]

The third benefit derives from the chemical composition of CNTs, which is very pure carbon. Carbon has already been used in many implant devices, including artificial heart valves, and no adverse effect of such biomaterials on living organisms has been reported to date.[96] The following features of CNTs may be regarded as advantages:

(1) High biocompatibility
(2) High strength-to-weight ratio
(3) High tensile strength
(4) Forming flexible nanofibers
(5) High chemical reactivity
(6) Conferring increased strength and other favorable characteristics to other substances when combined with them
(7) Inducing slow but significant biodegradation
(8) Colored in black that is easily distinguishable and detectable using a light microscope

The fourth benefit is the excellent electrical, magnetic, and thermal characteristics of CNTs in biomaterials. In fact, studies have used CNTs (because of their electrical characteristics) for nerve regeneration[112,120−122] and muscle actuation,[123,124] and (because of their magnetic characteristics) for cancer treatment and DDSs.[55,125] Furthermore, CNTs (because of their high photoenergy absorption capacity and thermal conductivity) have been proven effective for cancer thermotherapy.[56,107,126−128]

As stated above, CNTs (unlike conventional materials) can serve a wide variety of functions in tissues and cells of living organisms. This great potential has stimulated research into the application of CNTs as biomaterials in many fields. Overall, CNTs can be viewed as a revolutionary tool that will advance the practice of medicine, imposing expectations that biomaterials will be the main field of application of CNTs.

2.2. Application to Cancer Treatment

Currently, the most vigorously studied application of CNTs biomaterials is to cancer treatment. A wide variety of methods have been used to treat various cancers.[55,129−133] Detection of foci as early as possible and administration of an effective treatment are of paramount importance in cancer treatment. CNTs are expected to lead to innovative therapeutic and diagnostic methods. Although many other ongoing studies are not included, the following is an overview of the applications of CNTs to cancer treatment that are currently attracting much attention. Thus, clinical application of CNTs is a very promising field of study.

2.2.1. Biomarkers and Imaging. There have been recent dramatic technical improvements in methodology for the early diagnosis of cancer, with remarkable advances being made in tumor marker tests and the diagnostic imaging of cancer. Even now, however, it is difficult to detect early asymptomatic cancer; cancer is often detected only in the terminal stage. Against this background, studies have been conducted to detect the expression of biomolecules in the initial stage of cancer using CNTs as biomarker detectors. The application of CNTs to the detection of a prostate cancer marker (PSA), colorectal cancer markers (CEA, CA19-9), and a hepatocarcinoma marker (AFP) has been reported.[134−137] Applicability is based on the small size of CNTs that facilitates distribution in living organisms, and some evidence showing direct detection of biomarkers in vivo has been reported.[138−141]

CNTs have been used in noninvasive imaging, including for highly sensitive detection of very small tumors, using single CNT molecules conjugated to contrast reagent for CT or MRI, a heavy metal (gadolinium, etc.), and an antibody with high affinity for cancer cells.[142−145] A study is also ongoing that examines the application of a heavy metal encapsulated by the aforementioned peapod CNT to cancer imaging.[146] The most investigated imaging application is MR molecular imaging, which is effective in early detection of cancer. Furthermore, studies on the use of CNTs for photoacoustic molecular imaging show that it enhances contrast and resolution necessary to in vivo imaging. High resolution using a blend of SWCNTs and fluorescent peptide as the contrast medium for photoacoustic imaging were obtained.[147] Tumor vascularization plays an important role in cancer development and metastasis. For this reason, noninvasive detection of vascularization activity is critical to cancer diagnosis and assessment of patient responses to cancer treatment. A wide variety of molecular targets relevant to tumor vascularization have been identified, and can be used for tumor vasculature targeting and imaging. A method of optical imaging using a new photoprobe with the optical properties of CNTs has been developed to facilitate visualization of vascularization events.[148,149]

2.2.2. Drug Delivery Systems for Cancer Treatment. Of the biological applications of CNTs, DDSs for cancer treatment have been the most vigorously investigated. In cancer chemotherapy, adverse drug reactions are problematic, sometimes making it difficult to deliver adequate amounts of drugs to target organs. Because of their very large specific surface area that can bind many molecules beneficial to cancer treatment, CNTs can be used for DDSs in cancer treatment[89,129,150,151] and have been used as a platform to facilitate targeted delivery of a drug, antibody, other protein or peptide, lipid, polysaccharide, etc. (Figure 3). For example, a highly efficient missile therapy consisting of a combination of a hydrophilic group, a monoclonal antibody to cancer cells, an anticancer agent, and other components has been reported.[117] Using a nanoscale vehicle such as CNTs, drugs can be delivered to cancer cells that could not otherwise be delivered by microscale

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vehicles.[152] This is because thus-functionalized CNTs can pass through the cell membrane via a mechanism for the cellular uptake of foreign substances, such as endocytosis. CNTs with attached peptides or ligand bind to specific receptors on the cancer cell surface, and enter the cancer cells where they release the therapeutic agent more safely and efficiently. A DDS can be described as ideal when it delivers the needed amounts of therapeutic agent to the target in a timely manner, and CNT-based DDSs have the potential to fulfill this requirement.[132,153,154]

SWCNTs coupled with a tumor-specific monoclonal anti-CD20 antibody (rituximab) intravenously injected into mice after intramedullary transplantation of a human B-cell lymphoma resulted in accumulation of SWCNTs in the lymphoma.[110,155] Other researchers attached a tumor-recognizing module to the surface of hydrophilic f-SWCNTs to specifically bond with cancer cells, and then a prodrug module of an anticancer agent (a taxoid with a cleavable linker) to the surface of hydrophilic f-SWCNTs. They showed that the cytotoxicity of this tumor-targeting DDS is mediated via intracellular migration, drug release, and intracellular activation.[153] Moreover, the application of CNTs to gene therapy (i.e., as carriers of genes to targeted cancer cells) has been studied.[156−160] Because CNTs-based platforms are infinitely variable and easily designable, they are expected to lead to groundbreaking cancer treatment systems.

Before thus applying CNTs for DDSs, their pharmacokinetics after topical or intravenous injection must be clarified. The disposition of intravenously injected CNT−drug composite has been examined extensively.[105,161−165] Factors that influence transport of the composite through the bloodstream include thickness, length, and flexibility of CNTs as well as changes in properties resulting from the binding of the drug. Of course, injection of CNTs-based DDS into the tumor site directly is a safer approach. Furthermore, the use of magnetized particles to facilitate efficient uptake of CNTs in cancer tissue has been studied. For example, treatment of lymph node metastasis by subjecting magnetic functionalized CNTs to a magnetic field to promote their migration to lymph nodes has been studied.[166,167] Treatment with gemcitabine (GEM)-loaded magnetic functionalized CNTs subjected to a magnetic field resulted in regression of lymph node metastasis and suppression of metastatic growth both in vitro and in vivo.[55] In addition, many anticancer agent-loaded CNTs-based nanoscale DDSs have been developed.[101,128,168−170]

2.2.3. Cancer Treatment Using External Energy. CNTs absorb electromagnetic wave energy. On the basis of this property, the use of CNTs in cancer hyperthermia has been tested.[53,171−174] For example, cancer lesions were exposed to CNTs loaded with a tumor-specific epitope (to be absorbed selectively), then to infrared rays, and cancer tissue was specifically destroyed by the heat generated.[107] Another report showed the method for treating peritoneal metastases from colorectal cancer consisted of rapidly heating the cancer mass to 42 °C within 10 s in the presence of oxaliplatin or mitomycin C using infrared rays absorbed by CNTs.[175] In a recently reported study, the generation of heat and reactive oxygen species generated upon exposure of CNTs to infrared rays for 10 min was harmful to human lung cancer cells. Specifically, 45% of the cancer cells had been killed 24 h later.[56] The microwave absorption characteristic of CNTs theoretically permits accurate heating; microwave thermotherapy for cancer treatment is also a promising technology.[173]

Meanwhile, various improvements have been made in the targeting methods. Using anti-CD22 antibody coupled with SWCNTs followed by exposure to laser radiation succeeded in shrinking B cell lymphoma.[176] A study proposed that cancer cells could be destroyed using bubbles generated by administering CNTs and ethanol and exposing the cancer cells to laser light.[177] Recently, a nanosecond pulse electrical field was used to kill the pancreatic cancer cell line PANC1 in the presence of MWCNTs and resulted in a 2.3-fold reduction in cell survival as compared to control cells.[178]

In other studies, effect of thermotherapy was mediated through a CNT/DNA/IgG antibody composite bound to target cancer cells,[179] and the effectiveness of a CNTs/polyethylenimine/siRNA composite was attributable to RNA interference and photothermal therapy.[128] Furthermore, cancer imaging and thermotherapy was carried out concurrently by conjugating quantum dots to CNTs.[180] The variety of CNT applications has been increasing.

CNT peapods encapsulating iron nanoparticles and a chemical modification that facilitates binding to cancer cells have been used in cancer thermotherapy. The iron in the CNTs is highly biocompatible because it is protected from reacting with the ambient environment, and the electromagnetic wave thermotherapy is safe and effective.[118] In conclusion, investigations of thermotherapy with CNT adducts of other materials are ongoing.

These cancer treatments based on the ability of CNTs to absorb external energy cannot be clinically applied before methods of electromagnetic wave exposure are investigated. This is because the body rapidly absorbs the energy. In the case of simple exposure, the utility of CNTs is limited to accessible cancers. However, when used in combination with an implanted energy source, the utility of CNTs extends to deep cancers.[171,181,182] Cancer thermotherapy involving the clinical application of CNTs is currently a rapidly growing field of research.

2.3. Application to Regenerative Medicine

The aim of regenerative medicine is repair and regeneration of human body tissues and organs affected or lost because of disease, trauma, and the like. Developments in embryonic stem cell (ES cell) research and the development of induced pluripotent stem cells (iPS cells) in 2007 further stimulated regenerative medicine research.[183,184] Tissue regenerative therapies use cells, growth factors, genes, etc. Whichever means is used, no tissue can be regenerated without a scaffold. Thus, the scaffold is of paramount importance in therapy, and research aimed at developing CNTs as scaffold material has been increasing.[185−191]

2.3.1. Studies Assessing the Applicability of CNT Composites to Regenerative Medicine. The use of CNT composites in regenerative medicine has been vigorously investigated in vitro. Results showed that a CNT/collagen composite could be used as a scaffold for myocyte culture, and that a CNT/polyurethane composite could be used as a scaffold for fibroblasts growth and biosynthesis.[192−194] A CNT/polyurethane composite used as a scaffold for culturing vascular endothelial cells was effective in promoting their proliferation and suppressing thrombus formation.[195] A CNT/poly L-lactic acid/hydroxyapatite composite increased the adhesion and proliferation of periodontal ligament cells (PDLCs) by 30%.[196] Regenerated silk fibroin films incorporating MWCNTs were shown to support the adhesion and growth of human bone

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marrow stem cells.[197] SWCNTs nonwoven films enhanced long-term proliferation of many cell types.[198] While in vitro studies examining the reactions between cells and CNT composites used as scaffolds are numerous, there are few in vivo studies.[125,174,185−188,190,199,200] It is hoped that in vivo animal experiments based on in vitro findings will be carried out in the future. The application of CNTs to bone tissue regeneration and nerve tissue regeneration is of paramount interest.

2.3.2. Bone Tissue Regeneration. Regarding bone tissue regeneration, a CNT/polylactic acid composite was shown to promote osteoblast proliferation in vitro as early as in 2002.[58,201] Later, a CNT/polycarbonate urethane composite and a CNT/poly lactic-*co*-glycolic acid composite were reported to enhance the adhesion of osteoblasts.[202−204] In 2006, a study showed that SWCNTs and MWCNTs promoted the proliferation of osteocytes and osteoblasts when used alone.[205] This was followed by in vitro studies showing the wonderful effects of CNTs on bone-related cells.[66,186,206−212]

In 2008, we showed for the first time that CNTs promote bone tissue formation in vivo as well.[213] The study employed an experimental system that used recombinant bone morphogenetic protein-2 (rhBMP-2) to induce ectopic osteogenesis in mouse back muscle.[214] Bone formation on a collagen sheet was shown to occur earlier in the presence rhBMP-2 attached to a scaffold of MWCNTs than in the presence of rhBMP-2 alone (Figure 4). Later, other researchers confirmed that osteogenesis was promoted by CNTs in vivo. For example, a layer-by-layer assembled carbon nanotube composite promoted osteogenesis and bone repair when implanted in rat calvarial bone defects.[215] Carbon nanohorns, a type of CNT, were attached to a porous polytetrafluoroethylene membrane by vacuum filtration, and rat calvarial bone defects were covered with the membrane. The extent of osteogenesis was greater under the membrane containing carbon nanohorns than under the membrane without the carbon nanohorns, showing that carbon nanohorns accelerated bone regeneration.[216]

Later, we attempted to elucidate the mechanism underlying promotion of bone tissue regeneration by CNTs. In 2009, we showed that CNTs specifically suppressed the differentiation of osteoclasts as well as expression of the transcription factor NFκB in osteoclasts.[217] In 2011, we showed that CNTs could serve as the seed material for the crystallization of hydroxyapatite, the major component of bone, and that CNTs attracted Ca ions and activated osteoblasts. Another finding was that this activation was accompanied by the deposition of hydroxyapatite around the CNTs, which was catalyzed by alkaline phosphatase (ALP) released from osteoblasts.[218] These findings demonstrated that CNTs functioning as a scaffold interact with the body to promote osteogenesis and thereby the process of bone tissue regeneration. To date, no other scaffold has interacted with the body in this way; CNTs are expected to be breakthrough materials in regenerative medicine research as well.

2.3.3. Nerve Tissue Regeneration. Currently, brain injuries, spinal cord injuries, and large-gap peripheral nerve defects are intractable, and their treatment is an important goal of regenerative medicine. To enhance and stimulate the regeneration of these injured nerve cells and fibers, application of a wide variety of nerve conduits and synthetic guidance devices has been attempted but has failed to yield satisfactory results.[219] Applying CNTs is expected to lead to the development of new methods of nerve regenerative medicine and contribute to improvements in patient quality of life.[122,220−224]

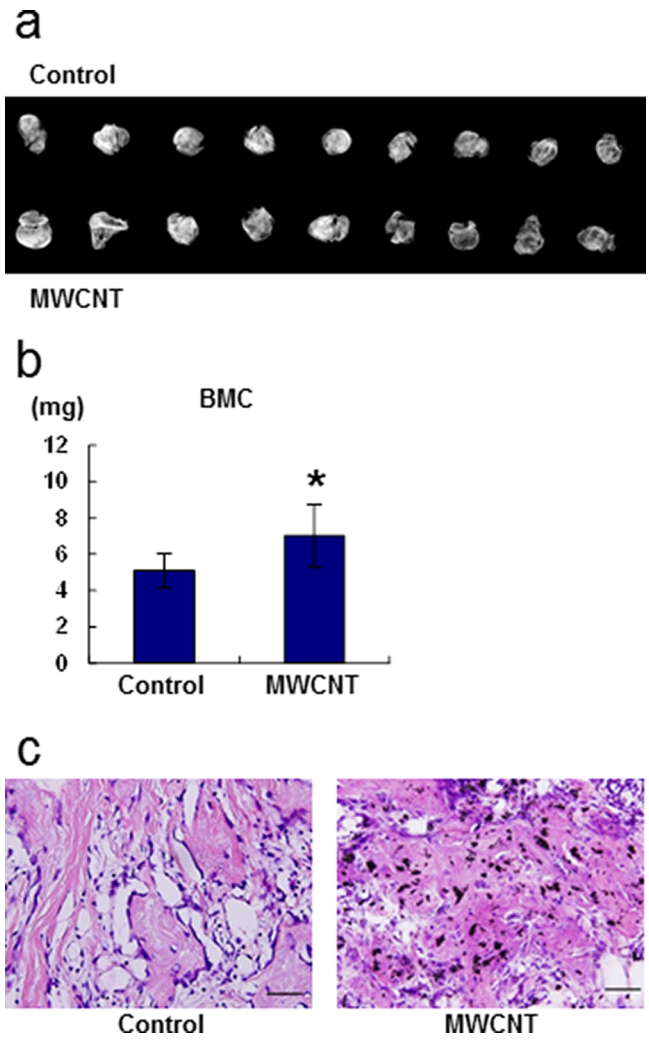

Figure 4. MWCNTs promote ectopic osteogenesis by rhBMP-2 and collagen. (a) A soft X-ray radiogram of newly formed bones extirpated 2 weeks after placement of rhBMP-2/collagen/MWCNT composite (upper lane) or rhBMP-2/collagen composite (lower lane) in mouse back muscle. Larger bones with more intense opacity were formed when using collagen conjugated with MWCNTs than without. (b) Bone mineral contents (BMCs) in bones formed at 2 weeks of implantation. A significantly higher BMC was observed in bones formed at 2 weeks of implantation of collagen conjugated with MWCNTs than without. Each error bar indicates the standard deviation of the mean (n = 8); asterisk, P = 0.016 between samples treated with carbon nanotubes and those without (unpaired Student's t test). (c) Histological images of bones extirpated at 2 weeks. The trabecula was thicker and denser when using collagen conjugated with MWCNTs than collagen alone. The tissue around the implanted collagen−MWCNT conjugate was found to have MWCNTs absorbed uniformly in the trabecula and bone marrow. The MWCNTs were seen to have entered the trabecula and came in direct contact with bone substrate. Hematoxylin-eosin staining. Scale bars = 100 mm. Reprinted with permission from ref 213. Copyright 2008 John Wiley & Sons, Inc.

Use of CNTs as a scaffold for neural cell growth has been vigorously studied for more than 10 years and found to be useful for neural cell adhesion and axonal growth.[117,225−227] CNTs promoted neurite elongation in a wide variety of cultured neurons.[228−230] CNTs were also reported to aid

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regeneration of Schwann cells.[231] Another study found that CNTs were useful in the differentiation of embryonic stem cells to nerve cells.[220] In these studies, electric stimulation was often used to promote neural cell growth, making the best use of the favorable electroconductivity of CNTs.[120] Regenerative medicine for nerve is an interesting research field that aims to apply in combination the electrical and mechanical properties of CNTs to biomaterials.

2.3.4. Regeneration of Other Tissues. The application of CNTs to the regenerative medicine of tissues other than bone and nerves has also been investigated. Cartilage regeneration was promoted by a composite of CNTs and polycarbonate urethane.[232] Other studies have examined the application of CNTs to skeletal muscle regeneration[233,234] and heart muscle regeneration. For example, inducing differentiation of mesenchymal stem cells to cardiomyocyte lineage cells by electrical stimulation with CNTs was succeeded in vitro, and notably finding evidence of electrically stimulated cross talk among these cells.[235] CNTs also promoted heart muscle maturity and altered the electrical characteristics of heart muscle.[236]

In the future, CNTs will be used to stimulate the regeneration of many other tissues and organs. Regenerative medicine is a field of applied medicine that capitalizes on the unique features of CNTs such as nanoscale size, large specific surface area, and high surface reactivity, as well as electroconductivity. Furthermore, unexpected effects, such as the promotion of osteogenesis resulting from the interactions of CNTs with the body, may be found in a wide variety of tissues, so regenerative medicine is quite an interesting field of applied research.

2.4. Application to Implant Materials

Implant technologies have been used in many clinical settings, such as orthopedic surgery, dental and oral surgery, and craniofacial surgery. Artificial valves and artificial blood vessels have been used in heart and other surgeries. These implants are required to possess, in addition to mechanical characteristics such as strength and durability, high biological compatibility because they come in direct contact with living tissue.[237,238] Many types of orthopedic implants, in particular, have long been used in clinical settings in many patients. Examples include artificial joints used to treat osteoarthritis and rheumatoid arthritis, plates and screws used to treat bone fractures, and cages and rods used for interbody fusion. Hence, many different materials are used in orthopedic implants.[239−241] Metals are used for bone fracture treatment and in artificial joints, including stainless steel, titanium alloys, cobalt−chromium, and tantalum. Ceramics (mostly alumina and zirconia ceramics) are used in artificial joints and artificial dental pulp. Ultrahigh molecular weight polyethylene (UHMWPE) is used in the sliding parts of artificial joints. Polyether ether ketone (PEEK) is often used for interbody fusion.

Since 2003, we have been working to conjugate CNTs to polyethylene for use in sliding parts and rotating parts of artificial joints.[58,69] The sliding parts of a polyethylene artificial joint wear away with long-term use, leading to the breakage of the artificial joint and necessitating revision surgery.[242−245] With this in mind, we are developing more durable artificial joints made of polyethylene and CNTs to reduce the amount of wear loss. The sliding parts of artificial joints are sometimes made of ceramic instead of polyethylene. Although ceramics are generally unlikely to wear, alumina ceramics break easily, and zirconia ceramics are liable to deform due to phase transition in vivo.[246,247] Hence, we are working to develop a new ceramic material (alumina ceramics combined with CNTs) that is unlikely to break down and deform.[248,249] Although many difficulties exist, including homogenously blending CNTs and ceramics, we have already obtained a blend with improved fracture toughness values. The number of patients undergoing artificial joint replacement surgery has been increasing each year worldwide; accordingly, the number of patients undergoing revision surgery is increasing steadily.[250] Clinical application of CNT-based artificial joints would dramatically reduce the number of patients undergoing revision surgery and allow use of artificial joints by young patients.

Furthermore, we are developing a CNT/PEEK composite for spinal fusion cages used in interbody fusion surgery. Spine interbody fusion cages of PEEK material have already been used clinically; however, poor bone compatibility poses an obstacle to the bonding of the implant and bone around it.[251,252] Hence, spine interbody fusion cages made of a CNT/PEEK composite of high bone compatibility are being developed by conjugating CNTs to PEEK, thereby utilizing the bone induction potential of CNTs described in section 2.3.2: Bone Tissue Regeneration. The development of these artificial joints and spine interbody fusion cages is further described in section 6.4.

In these composites, the CNT content ratio is up to 10 wt %, often about 5 wt %, with only a small amount of CNTs entering the body. Furthermore, because they are composite materials, there is little or no possibility that CNTs (that is particulate) will be directly exposed to living organisms. For this reason, CNT composites can be thought to be highly safe, with the reactions between CNT particles and living organism rarely posing a problem. In view of biological safety of CNT composites, we believe that the first application of CNTs should be in implants in the form of composites as described above.[253−255]

Taking into account the above-described utility of CNTs as a reinforcing material and their safety as composites, it is expected that a wide variety of CNT composite implants will be developed in the future. Although technically difficult, conjugating CNTs to metals and ceramics would produce great benefits. While this field has so far received only scant attention, we hope that more R&D effort will be directed to this field, where CNTs are most likely to find clinical applications.

2.5. Application to DDSs for Treatment of Noncancer Diseases

As stated in section 2.2.2: Drug Delivery Systems for Cancer Treatment, CNTs have large specific surface areas, possess high surface reactivity, and therefore can be conjugated with a wide variety of molecular species, including low-molecular-weight compounds, genes, proteins, and vaccines, in large amounts. In addition, because CNTs can be delivered to the small structures in living organisms, they are expected to act as an ideal DDS.[100,117,256−258] Research has recently been advancing rapidly toward the development of more useful CNT-based DDSs for various diseases. Many improvements have been made in the reactivity with the cell membrane, which is particularly important to DDS applications. For example, SWCNTs bound to an integrin monoclonal antibody were used to enhance their adhesion to cells.[114] Bonding of a bilayer-forming lipid to CNT surfaces was used to lessen the influence

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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.

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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.

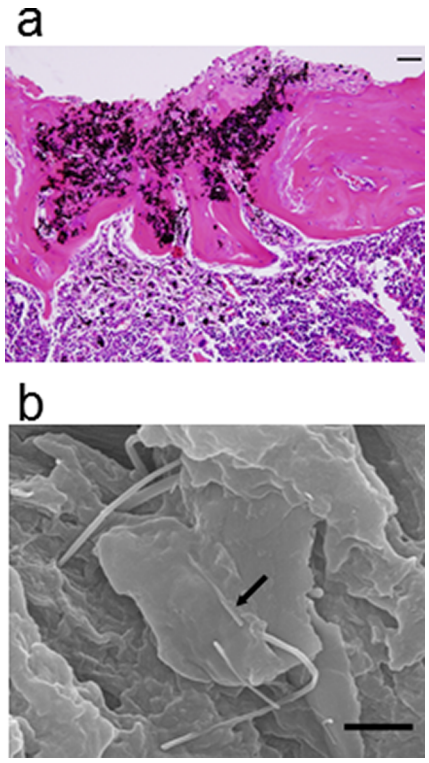

Figure 5. MWCNTs exhibiting good bone compatibility as they are absorbed in repaired bone without interfering with bone repair. (a) A histological image of a tibia extirpated 4 weeks after surgery for implant of MWCNTs in a pit drilled in tibial diaphysis after incising the anterior surface of a mouse 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

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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

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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.

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 ≤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]

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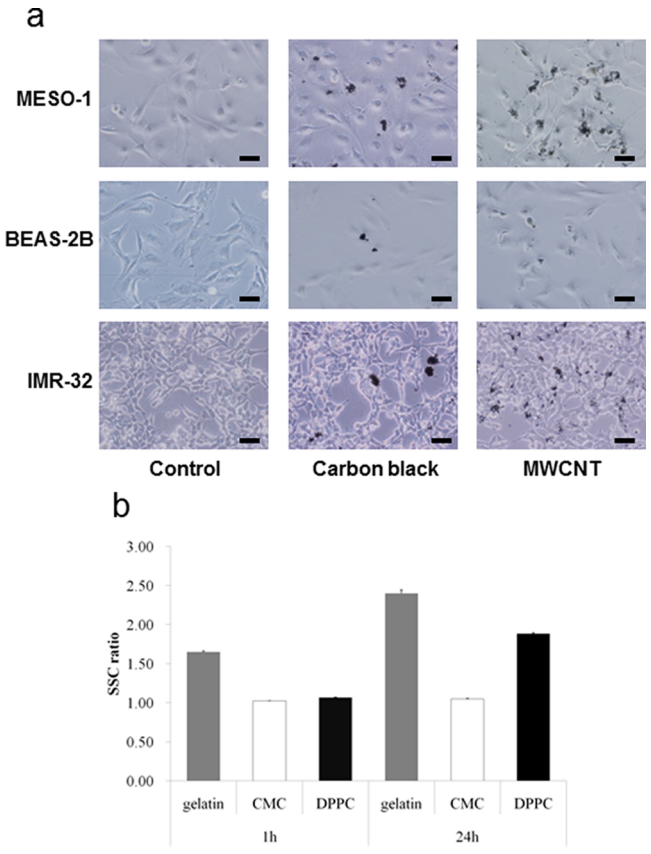

Figure 6. Cellular uptake of pristine MWCNTs varies depending on the type of cell and the choice of dispersant. (a) Combined images from bright field images and phase-contrast photomicrographs obtained 24 h after exposure of human malignant pleural mesothelioma cells (MESO-1), human bronchial epithelial 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

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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.

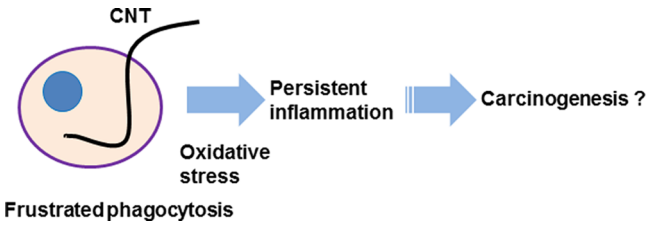

Figure 7. A schematic diagram showing a hypothesized mechanism of carcinogenesis due to frustrated phagocytosis. If left unabsorbed, long CNTs in cells can produce oxidative stress and induce inflammation. It has been suggested that a long period of persistent inflammation in the thoracic cavity following 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]

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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

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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.,

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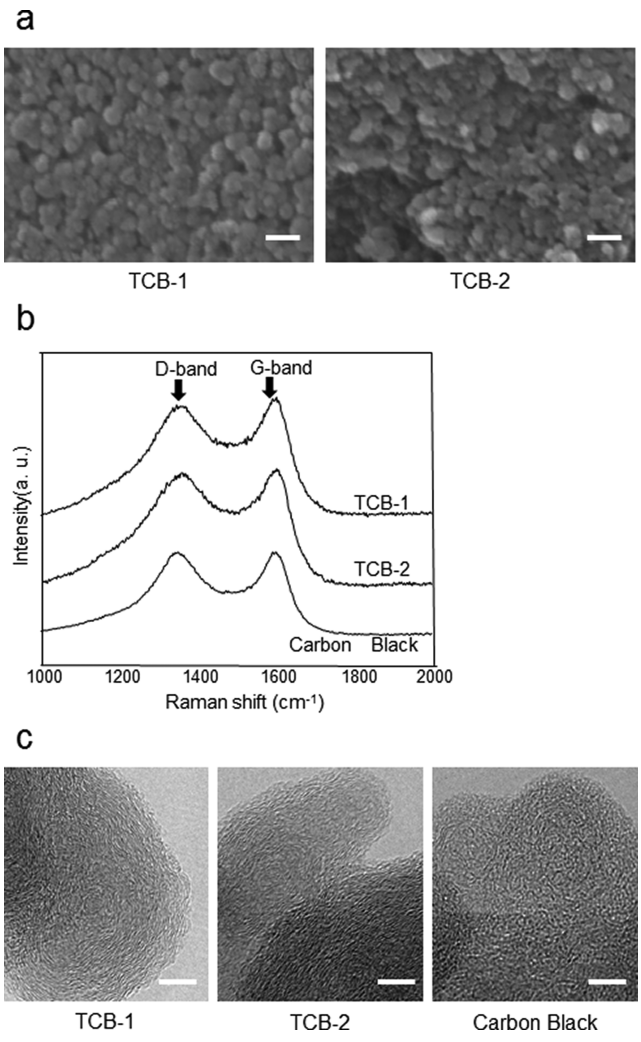

Figure 8. Having historically been proven safe to the human body, tattoos comprise nanosized highly pure carbon black, and hence serve well as a reference material for evaluating the safety of CNTs, which likewise occur in the form of nanosized carbon particles. (a) Scanning electron microscopy (SEM) images of tattoo carbon black-1 (TCB-1) and tattoo carbon black-2 (TCB-2) prepared by drying two different tattoos. TCB-1 and TCB-2 were found to have accumulated in the form of generally regular particles having a diameter of about 30−50 nm, and generally irregular particles having a diameter of about 50 nm, respectively. (b) Raman analysis of TCB-1, TCB-2, and ordinary carbon black. TCB-1 and TCB-2 exhibited nearly the same Raman shift pattern as with ordinary carbon black. D-band, turbostratic amorphous; G band, graphite crystal. (c) Transmission electron microscopy (TEM) images of TCB-1, TCB-2, and ordinary carbon black. These three substances were found to have nearly the same particle shape. Reprinted with permission from ref 97. Copyright 2011 Elsevier.

carbon black]) were compared to those of CNTs per se (Table 2).[476] Both substances are highly pure carbon particulates of similar size (i.e., they are nanosubstances, entities internationally recognized as being not less than 100 nm in one or more of the three dimensions).[477,478] CNTs and carbon black have distinct shapes: fibrous particles and spherical particles, respectively. Although various classifications of surface chemistry are available, the most common practice is to characterize surfaces as hydrophilic or hydrophobic. The surfaces of CNTs are hydrophobic, and carbon black particles, without surface treatment, are essentially hydrophobic. Hence,

Table 2. Comparison of Characteristics of CNTs and Carbon Black

characteristic	CNT	carbon black
composition	high-purity carbon	high-purity carbon
size	nanosized	nanosized
shape	fibrous particle	spherical particle
surface chemistry	hydrophobic	hydrophobic

three of the four representative characteristics of particulate substances are shared; therefore, it is reasonable to use carbon black as a reference for CNTs. Although some researchers may disagree based on the distinction between fibrous and spherical particles, no reference can have exactly the same characteristics as the test substance. Considering the absence of any other appropriate reference, it is very fortunate that carbon black with high similarity to CNTs has a long history of use in living organisms and demonstrated safety in the human body. Problems stemming from the fibrous nature of some nanoparticles are discussed in section 6.2. Despite these problems, it can be concluded that fibrous nanoparticles pose no hazard at sites of CNTs implantation if inflammation is not persistent.

All experiments can be performed using mass as an index because CNTs and carbon black are both highly pure carbon. For particulate substances, it is often difficult to measure the number and volume of particles; therefore, the ability to use mass as the simplest index is an obvious advantage in the evaluation of CNTs safety. Carbon black can also serve as a reference for the evaluation of the biological safety of other nanosubstances used as biomaterials. However, mass cannot be used as an index for safety comparison when density varies; therefore, another index such as particle count will have to be used, making the procedure more complicated and difficult to perform, and even reducing its accuracy.

Research into application of non-CNT carbon-based nanomaterials to biomaterials has also progressed steadily, although there are fewer non-CNTs studies than CNTs studies. The use of fullerenes or graphene for DDSs and imaging has been studied, and their biological safety has been evaluated.[479,480] Additionally, nanosized carbon fibers, which traditionally have not been nanosized, are now available thanks to recent technical advances. Although carbon fiber products are promising candidates as nanobiomaterials because of their history of clinical use as biomaterials, their safety needs to be evaluated because of their nanosize,[214,481] and carbon black can serve as an appropriate reference.

5.3.3. Safety Test. A skin implantation test with MWCNTs was conducted using carbon black tattoo ink as a reference material. Results showed that MWCNTs induced acute but mild inflammation reactions in subcutaneous tissue, which resolved early. The subcutaneously implanted MWCNTs were shown to be absorbed initially by macrophages and remained in the macrophages for a long time. These short- to long-time histological reactions to the MWCNTs were found to be very similar to the histological reactions to the carbon black tattoo ink particles (Figure 9). This finding shows that when implanted in vivo, MWCNTs (as with tattoo ink particles) exhibit good tissue affinity at the implantation site and stay intact in macrophages for a long time.[97]

We then conducted a colony formation assay to determine the in vitro cytotoxicity of MWCNTs using carbon black tattoo ink particles as a reference. Both MWCNTs and carbon black tattoo ink particles inhibited colony formation in a concen-

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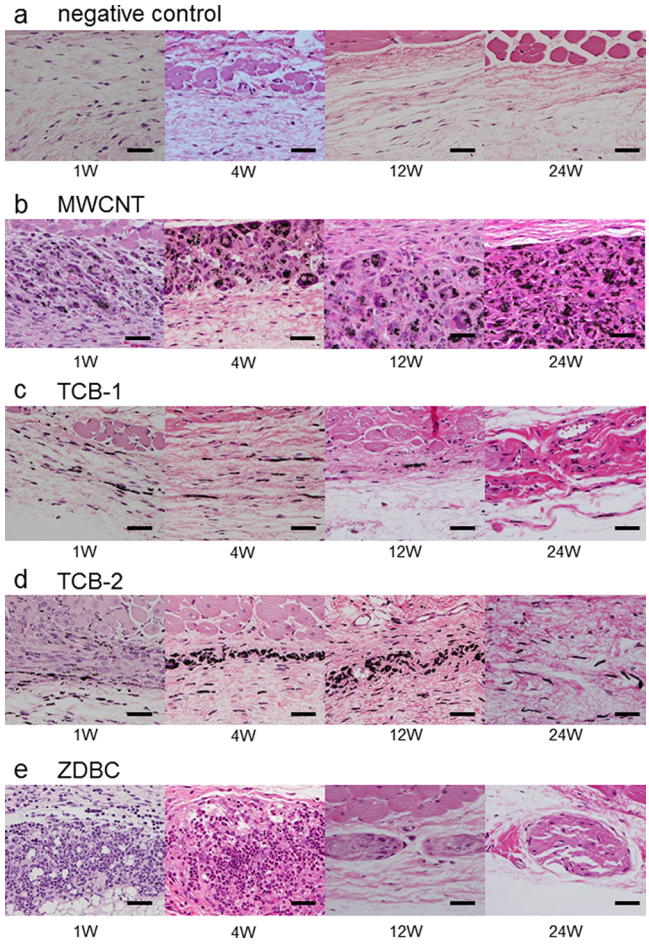

Figure 9. Histological reactions to subcutaneously implanted MWCNTs are very similar to those to carbon black, showing good tissue compatibility. Hematoxylin-eosin staining. Scale bars = 20 μm. TCB-1, Tattoo carbon black-1; TCB-2, Tattoo carbon black-2 (see Figure 8). (a) Histological images from a negative control group (NC) of male ddY mice at 6 weeks of age receiving an injection of 10 μL of physiological saline and a surfactant given to a pocket created in subcutaneous tissue in the back. At 1 week of treatment, the subcutaneous tissue had been repaired nearly completely. Repair was complete at 4 weeks. No change was observed at 12 and 24 weeks. (b) Histological images of subcutaneous tissue from a group receiving an injection of 10 μL of MWCNT solution (4.0 mg/mL). Most particles were found to have been absorbed in macrophages at 1 week. In the areas around the injection site, accumulated fibroblasts, neutrophils, and lymphocytes were found, with weak inflammatory reactions observed. At 4 weeks, the MWCNTs remained incorporated in macrophages, and the inflammatory reactions around the injection site had resolved. The macrophages that had absorbed MWCNTs turned into multinucleated giant cells, creating an appearance like foreign-body granuloma. The histological profiles obtained at 12 and 24 weeks did not differ from the profile obtained at 4 weeks. (c) Histological images of subcutaneous tissue from a group receiving an injection of 10 μL of TCB-1 solution (4.0 mg/mL). At 1 week, most particles were found to have been absorbed in macrophages in the subcutaneous tissue, and as in the MWCNT group, accumulated fibroblasts, neutrophils, and lymphocytes were found, with weak inflammatory reactions observed. At 4 weeks, the inflammatory reactions around the injection site had resolved as in the MWCNT group. The histological profiles obtained at 12 and 24 weeks were similar to the profile obtained at 4 weeks. (d) Histological images of subcutaneous tissue from a group receiving an injection of 10 μL of TCB-2 solution (4.0 mg/mL). All histological profiles obtained at 1, 4, 12, and 24 weeks were similar to those obtained with the TCB-1 solution. (e) Histological images of subcutaneous tissue from a group receiving an injection of 10 μL of zinc dibutyldithiocarbamate (ZDBC) solution (4.0 mg/mL). At 1 week, accumulation of many types of inflammatory cells such as fibroblasts, neutrophils, lymphocytes, and plasma cells was observed, and intense inflammatory reactions had been induced over a wide area, with fat necrosis and nuclear debris formation observed. No accumulation of macrophages was observed. Even at 4 weeks, inflammatory cells remained and inflammatory reactions persisted, although the inflammation was going to disappear. At 12 and 24 weeks, the inflammatory reactions had resolved, and the subcutaneous tissue had been repaired into scar tissue with fibrosis. Reprinted with permission from ref 97. Copyright 2011 Elsevier.

tration-dependent fashion. At higher concentrations, colony counts were higher with exposure to MWCNTs than with exposure to carbon black tattoo ink particles (Figure 10). These findings demonstrated that the cytotoxicity of MWCNTs was not greater than that of carbon black tattoo ink particles.[97] When assessing the cytotoxicity of nanoparticles, the colony formation assay yields numerical results, and is currently considered to be the best (most sensitive and reproducible) method of toxicity assessment.[402]

Furthermore, we conducted a carcinogenicity test of CNTs in a trancegenic rasH2 mouse[482−485] using tattoo carbon black tattoo ink as a reference material. The rasH2 mouse has recently also been used in studies of bulk biomaterials.[486−488] We implanted MWCNTs or tattoo carbon black subcutaneously. Results showed that no neoplasms were produced because of implantation of MWCNTs. In the group with carbon black implanted as a reference, one animal died but had apparent tumors on histopathological examination (Figure 11, Table 3). The 75 mg/kg dose of MWCNTs implanted in this study was considerably higher than the doses that had been used in previous implantation studies in ordinary mice.[89,91,155,304] In summary, the above-described test for assessing the carcinogenicity of subcutaneously implanted CNTs by in the trancegenic animals for the first time revealed no carcinogenesis from CNTs as well as tattoo carbon black tattoo ink.[98]

The aforementioned tests showed that nanosized carbon black particles (a tattoo ink component) could be used as a reference for safety evaluation of CNTs. The in vivo implantation test, cytotoxicity test, carcinogenesis test (in transgenic mice), and other tests all found that the toxicity of CNTs is equal to or less than that of carbon black tattoo ink. If a safety test using a substance with verified biological safety as a reference material finds that CNTs exhibit a level of toxicity equivalent to, or lower than, that of the reference material, then it can be concluded that CNTs are safe. We are currently conducting mutagenesis and genotoxicity tests with highly pure carbon black as a reference material. So far, our results show that CNTs are as safe as carbon black particles under the experimental conditions used in the studies.

6. DISCUSSION AND PERSPECTIVE

6.1. Available Safety Evaluations Relevant to CNTs as Biomaterials

In this Review, studies on using CNTs as biomaterials have been reviewed, and currently available in vivo and in vitro studies on the evaluation of CNTs safety as biomaterials have been described separately. It is clear that many benefits will

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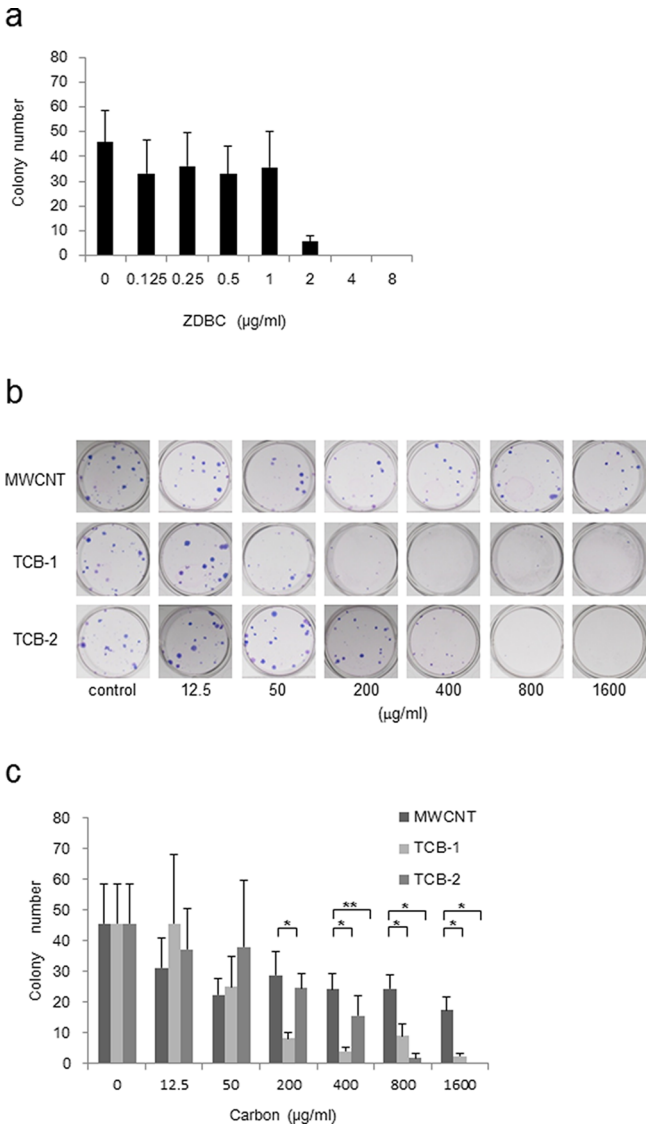

Figure 10. The cytotoxicity of MWCNTs is not higher than that of carbon black. TCB-1, Tattoo carbon black-1; TCB-2, Tattoo carbon black-2 (see Figure 8). (a) Appropriateness of cytotoxicity assessment in colonization test. The colonization capacity of V79 cells (Chinese hamster lung fibroblast cell line JCRB0603) decreased as the concentration of the positive control ZDBC increased. The concentration for 50% colony count reduction (IC_{50}, reference value range: 1−4 μg/mL) was found to be between 1 and 2 μg/mL, confirming that the cytotoxic action of the test substance was properly assessed. (b) Macroscopic photographs showing colonization test results. Colony counts of V79 cells cultured using a culture broth alone and those cultured in the presence of MWCNT solution, TCB-1 solution, and TCB-2 solution were compared. The concentrations in the solutions were 12.5, 50, 200, 400, 800, and 1600 μg/mL, respectively. (c) Colony counts versus carbon concentrations in MWCNT, TCB-1, and TCB-2 solutions. MWCNTs inhibited the colonization in a concentration-dependent fashion, and TCB-1 and TCB-2 likewise inhibited the colonization in a concentration-dependent fashion. When comparing colony counts, MWCNTs produced significantly higher colony counts than TCB-1 at concentrations of 200 μg/mL or more, and than TCB-2 at concentrations of 400 μg/mL or more. Error bars indicate standard deviations (n = 6); *, p < 0.001; **, p = 0.016. Reprinted with permission from ref 97. Copyright 2011 Elsevier.

come from application of CNTs biomaterials to a wide range of important medical services, including cancer treatment, regenerative medicine, implants, and DDSs.[180,489−491] Making the best use of the findings of these application studies would improve current clinical practices and ensure remarkable progress of medical care. For this reason, research into application of CNTs to biomaterials has been increasing rapidly (Figure 1); however, no clinical application of CNTs has been realized yet[77] because the evidence for the biological safety of CNTs as biomaterials is not definitive. It is easy to say that certain new materials pose risks to living organisms; when one study raises a safety-related question, the test substance can be said to be risky but cannot be said to be completely safe unless its safety is demonstrated in all situations where it is likely to be used. Safety cannot be assured without conducting numerous studies, and this is practically impossible. Therefore, as many studies as possible are needed to make a reasonable, acceptable consensus judgment based on a comprehensive assessment of the findings. It would otherwise be impossible to realize the clinical application of a new biomaterial. The accumulated research into the application of CNTs biomaterials is already sufficient to make such a judgment. The primary objective of this Review is to logically determine whether CNTs can be safely used as biomaterials in clinical settings.

Many pioneering studies have evaluated the safety of CNTs biomaterials. A wide variety of CNTs have been used in many different ways, and studied using various methodologies by many different researchers. Therefore, different studies have often yielded inconsistent results. However, the right judgment must be based on a comprehensive assessment of all such results. To this end, this Review has comprehensively reviewed the relevant published literature and described as many of the latest findings as possible. We conclude that the number of studies reporting the biological safety of CNTs as biomaterials is increasing and that most of the recently published reviews have concluded that CNTs are very safe.[91,99,191,326,492] Taken together, the findings suggest that CNTs will find clinical application as biomaterials through a stepwise process involving appropriate methods and sites of use.

6.1.1. In Vivo Studies. As stated above, no reported in vivo studies have found that CNTs are associated with life-threatening or otherwise serious toxicities such as carcinogenicity. Furthermore, recently reported studies for the most part have shown that inflammation in response to highly pure CNTs implanted in the body was not intense and resolved quickly.[58,89,91,155,304] The organ(s) sites of CNTs accumulation after transport through the bloodstream, the response of tissues and cells to the accumulation, and period of CNTs accumulation are important issues. No study has reported any problem resulting from intravenous injection of CNTs.[91,191,310] However, more accurate techniques must be developed for monitoring the behavior and disposition of CNTs intravenously injected in large amounts for use in DDSs and imaging. Bearing in mind that CNTs can enter the pulmonary circulation when inhaled, the development of such techniques is ongoing worldwide.[86,144,306,312−315] Hence, the distribution of CNTs after passage through the bloodstream will be revealed in the near future. At present, researchers should refrain from clinically applying CNTs to sites with abundant blood supply until adequate data are available to verify its safety.

On the other hand, it is necessary to determine whether CNTs topically used as biomaterials enter the bloodstream. Because CNTs are particles, they should be limited by size from

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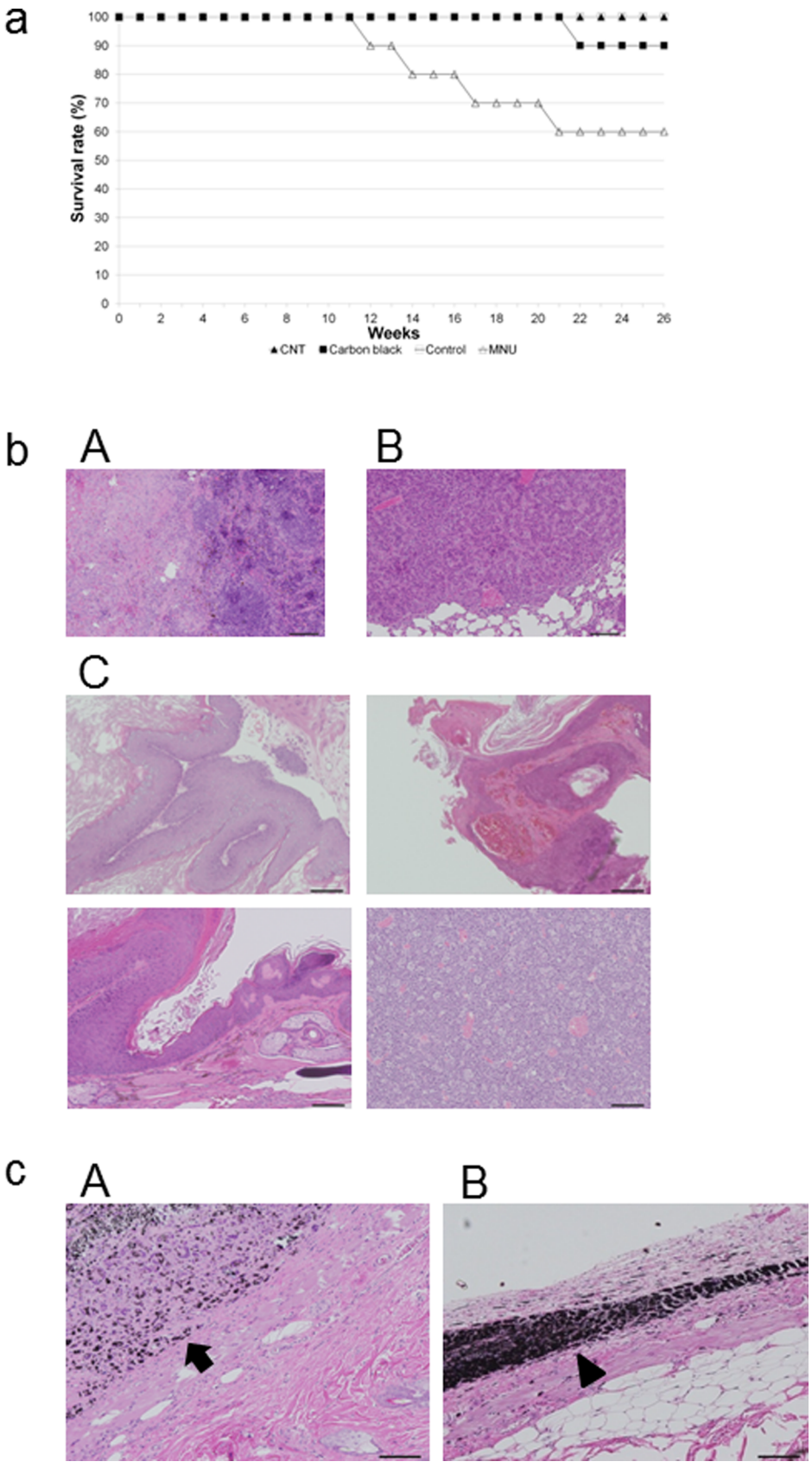

Figure 11. In a subcutaneous implantation test using cancer-developing transgenic rasH2 mice, MWCNTs were found to be not carcinogenic; their carcinogenicity was determined to be not higher than that of carbon black. (a) Changes over time in survival rate of rasH2 mice. All mice in the MWCNT group were alive at 26 weeks. In the carbon black group, 1 animal died at 22 weeks, and at 26 weeks, 9 of the 10 animals were alive. In the solvent group, all mice were alive at 26 weeks. In the *N*-methyl-*N*-nitrosourea (MNU) group, 1 animal died at 13, 14, 17, and 22 weeks each; 6 of the 10 animals were alive at 26 weeks. (b) Histological images of tumor masses in various organs of cancer-developing mice. (A) A tumor mass was

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Figure 11. continued

observed in the spleen of 1 of the 10 mice in the MWCNT group that survived for 26 weeks; this was identified as an inflammatory pseudotumor, not a neoplasm. (B) A neoplasm developed in a lung of 1 mouse in the carbon black group that survived for 26 weeks; this was diagnosed as a benign adenoma. (C) All 10 mice in the MNU group had tumors. Proventricular tumors developed in all 10 animals, with abnormal squamous epithelial growth observed (upper left panel). Skin tumors developed in 6 of the 10 animals, which occurred as malignant skin tumors in the thigh (upper right panel). Genital tumors developed in 4 animals (lower left panel). A thymic tumor developed in 1 animal (lower right panel). Hematoxylin-eosin staining. Scale bars = 10 μm. (c) Histological images of subcutaneous implantation sites taken at 26 weeks. (A) In the MWCNT group, no neoplasm developed, with macrophages found to have accumulated while phagocytosing MWCNT particles. No inflammatory cells such as neutrophils and lymphocytes were observed around the implantation sites. (B) In the carbon black group, like in the MWCNT group, macrophages phagocytosed MWCNT particles, with no neoplasm observed. Arrow, MWCNTs; arrowhead, carbon black. Hematoxylin-eosin staining. Scale bars = 10 μm. Reprinted with permission from ref 98. Copyright 2012 Nature Publishing Group.

Table 3. Neoplastic Changes in rasH2 Mice Implanted with CNT, Carbon Black, Solvent, or *N*-Methyl-*N*-nitrosourea (MNU) Solution[a]

		total number			
		control	carbon black	CNT	MNU
organ	diagnosis	10	10	10	10
skin (back area)	papilloma	0	0	0	2
	keratoacanthoma	0	0	0	0
skin (face)	papilloma	0	0	0	3
	keratoacanthoma	0	0	0	0
skin (thigh)	papilloma	0	0	0	1
	keratoacanthoma	0	0	0	0
spleen	inflammatory pseudotumor	0	0	1	0
	hemangioma	0	1	0	0
hematopoietic system	malignant lymphoma	0	0	0	2
	epithelial thymoma	0	0	0	0
kidneys	hemangioma	0	0	0	0
pancreas	hemangioma	0	0	0	0
lungs	adenocarcinoma	0	0	0	0
	adenoma	0	1	0	1
	hemangioma	0	0	0	0
forestomach	papilloma	0	0	0	10[b]
	basal cell tumor	0	0	0	0
	squamous cell carcinoma	0	0	0	0
perineal	papilloma	0	0	0	5[c]

[a]Adapted with permission from ref 98. Copyright 2012 Nature Publishing Group. [b]Significant differences at p = 0.0000054125 (Fisher's direct method). [c]Significant differences at p = 0.016254 (Fisher's direct method).

entering the bloodstream. MWCNTs above a certain size are thought to rarely enter the bloodstream.[310] Even if they enter from a local site, the concentration is expected to be too low to have a major impact on the body.[91,139,191,310]

In conclusion, on the basis of available in vivo data, it can be concluded that MWCNTs are likely to be useful topical biomaterials.[152] Of course, sites of intensive inflammatory reaction to MWCNTs and likely sites of MWCNTs entry into the bloodstream appear to depend on the organ and tissue where the biomaterials are implanted.[304] For this reason, investigations should be conducted for each site separately to determine the hazard to each organ and each tissue.

6.1.2. In Vitro Studies. The results from in vitro toxicity studies are difficult to interpret. Scientific international standards, typically ISO standards, have been established to assess the toxicity of bulk biomaterials,[467] but not nanoparticles like CNTs, which have distinct properties. In most recent studies, CNTs (adequately dispersed in solution) are regarded as chemical substances. This assumption seems to be reasonable for purposes of assessing the safety of particulate substances. In 2012, the Organization for Economic Co-operation and Development (OECD) announced, "Although most testing/assessing methods for conventional chemical substances are also suitable for nanomaterials, corrections according to the characteristics of nanomaterials may be needed in some cases."[493]

It should be noted that it is difficult to determine which of the many factors relevant to CNTs is being assessed in an assessment of in vitro CNTs toxicity. These factors include thickness, length, shape, surface reactivity, and aggregability as well as the influence of residual metals, dispersants, and assay reagents.[117,263,334] Because these factors are inter-related, their influences are difficult to determine separately.[494,495] Variation in the thickness and length of CNTs may be associated with their cytotoxicity. SWCNTs and MWCNTs differ in toxicity profiles; for example, SWCNTs are more likely than MWCNTs to induce oxidative stress. Length is also important; long CNTs are likely to induce oxidative stress because they are not completely phagocytosed by macrophages. The toxicity of inhaled CNTs increases with increase in length above 10−20 μm, although this observation has not been confirmed.[412] While the maximum acceptable length of CNT particles used as biomaterials is unknown, long CNTs are not considered to pose a problem (such as an inhalation problem), as stated in section 6.2. Surface reactivity also differs depending on the type of CNTs; furthermore, the toxicity of chemically modified CNTs should be thoroughly examined for each modification. Although the influence of residual metals is not negligible, many studies have found that CNTs with a quite high purity pose no major problem.[72,365,400−402,407,408,496] On the other hand, the influence of aggregability of CNTs and the choice of dispersant on toxicity do pose problems.[483] Many published studies are thought to have established the total toxicity of CNTs and dispersant.[371,373,385,497] Furthermore, because the aggregability of CNTs and the choice of dispersant vary widely among different studies, the range of CNT concentrations used in respective toxicity studies is wide (from 1 ng/mL to 10 μg/mL), making assessment more difficult.[309,447] From now on, in vitro studies should be conducted under a standard set of conditions (dispersant selected to not affect the test cells, and range of concentrations selected to avoid CNTs aggregation) whenever possible.

6.1.3. Correlations between in Vivo and in Vitro Data. For CNTs, unlike drugs and other chemical substances, little is known about correlations between in vivo and in vitro data.[498] One reason is that no in vivo data are available; nanoparticles

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have not yet been used as biomaterials, and this issue cannot be resolved until clinical application is realized. Another reason is that there are no standard ways for dealing with a wide variety of factors that can influence the results of correlation studies, such as the animal species, method of administration used in the in vivo studies, and the choice of cell and culture broth used in the in vitro studies.[91] In the future, it will be necessary to ensure these factors are consistent for all studies, to collect and analyze data from international sources, and to determine the correlations between in vivo and in vitro toxicity assessments of CNTs, at least in small animals such as mice.[191]

6.2. Appropriate References for Safety Evaluation of CNTs

6.2.1. Requirements for References. Only a few articles on the safety of CNTs biomaterials were published before 2010, and these reported many conflicting results. However, as the number of articles increased, the number of safety evaluations increased, and the conclusion drawn from these results is that CNTs are safe to use as biomaterials.[89,91,99,191] Nevertheless, clinical application has been frustrated because researchers have been unable to rule out CNTs toxicity. This situation is due primarily to the lack of a best reference material for the evaluation of CNTs safety. The finding of such a reference would facilitate evaluation of CNTs safety.[68] CNTs biomaterials, like all other biomaterials, are foreign to living organisms irrespective of their biocompatibility. At concentrations exceeding a certain level, CNTs exhibit toxicity in vitro and in vivo. The absence of a reference with established safety makes it difficult to determine the in vivo safety of CNTs. For example, when test cells lose activity in the presence of CNTs at concentrations exceeding a certain level, it is wrong to conclude that the CNTs are cytotoxic. However, it is right to conclude that CNTs are not cytotoxic when test cells lose activity in the presence of a reference with established safety. Biological safety cannot be assessed without comparison to a reference that has been proven to be safe in living organisms as described above. However, unfortunately, no such reference has been found to evaluate the safety of CNTs biomaterials. Inevitably, CNT toxicity studies have yielded inconsistent results so that no safety evaluation has been regarded as reliable. The lack of a reference with confirmed biological safety is attributed to the traditional view that nanoparticulates are not biomaterials.

6.2.2. Carbon Black. We found a reference material (carbon black) and proposed its use as a reference in our research articles published in 2011 and 2012.[97,98] Carbon black is the primary component of black tattoo ink, and tattooing of the human body has a long history dating back before ancient times, and is currently commonly performed.[475] Some researchers may dispute the use of carbon black as a reference for CNTs because CNTs particles are fibrous and carbon black particles are spherical. It is reasonable to attach importance to this difference if the research focus is on inhalation toxicity. Cells are unable to completely absorb long fibrous nanoparticles. It has been hypothesized that oxidative stress "frustrates" phagocytosis, and prolongs inflammation and other events.[281,409,411,412] However, this merely accounts for prolonged inflammatory reactions to CNTs in the thoracic cavity, where inhalation exposure to CNTs occurs, and in the abdominal cavity (a surrogate for the thoracic cavity), where exposure to CNTs is experimentally mimicked. Many researchers have found that even when a considerable amount of CNTs is implanted subcutaneously and elsewhere, only transient, very mild inflammation develops and resolves quickly.[58,307−309] This fact suggests that no frustrated phagocytosis occurs at least in subcutaneous tissue. Hence, because no in vivo implantation study found that CNTs cause frustrated phagocytosis at sites of transient inflammation, it can be concluded that fibrous nanoparticles pose no risk. Generally, animal experiments have shown the improbability that sites of prolonged CNT-induced inflammation contain CNTs particles. Therefore, provided that appropriately designed in vivo implantation studies produce no evidence of prolonged inflammation, then fibrous CNTs can be used as biomaterials without safety concerns.

Essentially, sharing all of the characteristics of the test material is not the only requirement for a substance to be used as a reference. This is also true for bulk biomaterials; even with different characteristics, they have been used as references with satisfactory results for desired effects.[467] Importantly, both CNTs and carbon black belong to a new category of biomaterials known as nanoparticulate substances. Logically, as with bulk biomaterials, no problem arises from the use of carbon black as a reference material for CNTs. Carbon black (like CNTs) is a nanosized particulate substance, even though other characteristics may be different. Another advantage of carbon black as reference is that mass can be used as an index because both CNTs and carbon black are pure carbon particulates.[97,98] Mass is by far an easier index to use in toxicity studies than particle count and volume. In view of these facts, we propose the use of highly pure carbon black as a good reference material for CNTs.

Many articles are available on the use of carbon black as a reference for assessment of biological reactions to CNTs.[292,365,381,499−506] However, no clear evidence has been presented supporting the claim that carbon black is safe to use in living organisms. According to many researchers who have used carbon black as a reference for CNTs, carbon black is intuitively the best reference. We have verified the scientific intuition of many researchers by providing them with a rationale (i.e., carbon black is safe because it is a component of black tattoo ink). Because there are a large number of such articles, we believe that many researchers will agree with the conclusion of this Review that carbon black is suitable as a reference material for safety evaluation of CNTs.

6.2.3. International Standards. What should happen soon after a consensus is reached that carbon black is suitable for use as a reference material for CNTs? The safety of CNTs should be evaluated both in vivo and in vitro using the new reference material. As stated above, it is easy to say, "Risk may exist", but it is difficult to say, "No risk exists". It is necessary for as many researchers as possible to conduct as many studies as possible. All studies then need to use standardized carbon black as a reference to allow the results to be assessed collectively and comprehensively compared.

There are many types of carbon black with somewhat variable biological safety. Above all, highly pure carbon black (a suspension of nanoparticles, which is equivalent to the carbon black used in tattoo ink) can be used as a reference for CNTs.[475] At present, we think that carbon black particles (diameter of about 50 nm and a purity of 99.5% or more) are suitable, but we would like to suggest here that many experts discuss extensively, choose, and designate the best carbon black powder as the international standard.

Special attention should be paid to the carbon black dispersant (usually a surfactant) because CNTs particles in

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Table 4. Stages of Clinical Application of CNT-Based Biomaterials[a]

atage	nature of the biomaterial	site of use	degree of in vivo exposure	risk	example of use
stage 1	composite	topical	none/low	none/low	artificial joints and interbody fusion materials
stage 2	particulate	topical	intermediate	low/intermediate	DDSs and imaging for cancer treatment
stage 3	particulate	topical	intermediate	low/intermediate	regenerative medicine scaffolds and DDS for topical treatments
stage 4[b]	particulate	systemic	high	high	DDSs and imaging that circulate via bloodstream

[a]Clinical application of CNTs to biomaterials should progress demonstrating the safety at each stage. [b]The decision of proceeding to stage 4 requires extremely careful consideration.

the test solution are less dispersible than carbon black particles.[366−371] The same dispersant should be used in both the CNTs and the reference solutions, provided the dispersant (when used at concentrations that fully disperse the CNTs and reference particles) has no major impact on living organisms and cells. In vitro, in particular, particles precipitate over time, which can alter the cellular reactions depending on the precipitation rate. To ensure a valid comparison with the reference, it is desirable to use a dispersant that minimizes precipitation of particles. At present, we think polyvinyl alcohol is the best dispersant.[97] However, there may be better dispersants with higher dispersion efficacy, lower toxicity, greater ease of handling, and other superior characteristics; therefore, an internationally acceptable dispersant should be chosen after much discussion on the basis of a consensus of expert opinions.

6.2.4. Method of Safety Evaluation. CNTs with equal or less toxicity than that of the reference carbon black should be considered "safe". This judgment can be made without further research. If the toxicity of CNTs is found to be greater than that of carbon black, the decision should be deferred. Strictly speaking, further assessment is impossible because carbon black is the only currently available reference. Particular attention should be paid if toxicity is far greater than that of carbon black (e.g., toxic concentrations one-tenth of the reference concentration). On the other hand, if neither CNTs nor carbon black is toxic, CNTs should be considered nontoxic, or the study conditions should be considered inappropriate. We propose to collect safety data through various evaluations of CNTs using carbon black as a common reference, while paying attention to these facts.

Unfortunately, carbon black cannot be used as a reference material in the assessment of in vivo kinetics because particle shape affects localized nanoparticle accumulation and nanoparticle migration from tissue to bloodstream.[327] As compared to CNTs particles (that are fibrous), carbon black particles (that are spherical) migrate more readily between tissues and the bloodstream. However, a reference is not needed to track the in vivo migration of CNTs. If CNTs accumulate in a certain organ, a study of CNT implantation may be conducted to assess the biological reactions at the site using carbon black as a reference.

6.3. Decision To Start Clinical Application of CNT-Based Biomaterials

As stated above, many researchers have shown that pristine (very pure) MWCNTs with few failures as biomaterials are very safe to use as biomaterials. MWCNTs are safe to use topically but not at special sites such as the lung and abdominal cavity.[91,191,305,306] The safety of using MWCNTs as DDSs or the like and involving access to the bloodstream has not yet been verified. Furthermore, using tattoo carbon black as a reference, we showed that pristine MWCNTs are at least as safe as carbon black.[97,98]

Because the above-described remarkable advances in research into the application of CNTs as biomaterials have led to the judgment that CNTs biomaterials are probably very safe (provided the method and site of use are appropriate), now is a time to start using CNTs clinically. We are planning to clinically apply MWCNTs (carbon purity, of 99.5% or more; mean diameter, about 60 nm [40−90 nm]; mean length, about 10 μm; and specific surface area, 25−30 m^2/g; produced using the chemical vapor deposition technique [MWNT-7, Hodogaya Chemical, Tokyo, Japan]). Of course, a composite material containing 5 wt % or less of MWCNTs (the safest form of CNTs) will be used.

6.4. Path to Clinical Application of CNT-Based Biomaterials

Importantly, we will begin with the safest clinical application of CNTs and proceed in steps according to the magnitude of risk involved. We divided the time course into four stages differing in degree of risk estimated on the basis of the nature of the biomaterial (composite versus particulate), the site of use (topical versus systemic), and the degree of in vivo exposure to the particles (high versus low) (Table 4).

The first stage is characterized by the use of a CNTs composite material for implantation (stage 1). Generally, the CNT content in a composite material is not more than 10 wt %, and the likelihood of in vivo exposure to CNTs particles is zero or minimal. Therefore, problems due to CNTs in the human body are unlikely to occur. As the first biological application of CNTs, we are planning to use composites of MWCNTs with existing biomaterials in artificial joints or spine interbody fusion materials.

In application to artificial joints, we are developing an MWCNT/polyethylene composite material and an MWCNT/ceramics composite material. Although the polyethylene used in sliding parts of artificial joints is ultrahigh molecular weight polyethylene (UHMWPE), it wears during long-term use and can necessitate resurgery.[242−245,507] For this reason, cross-linked UHMWPE has become commonly used, although its excessive hardness and easy breakability are problematic.[508−512] Having favorable characteristics that are absent in conventional materials, that is, high wear resistance and low breakability, MWCNT-conjugated UHMWPE is suitable as a sliding parts material for artificial joints (Figure 12). On the other hand, ceramics are also used in the sliding parts of artificial joints. Although ceramics wear very slightly, they are breakable so that resurgery is sometimes needed.[513−518] Combining CNTs with ceramics increases fracture toughness and can transform ceramics into an ideal, wear-free, antifracture, sliding parts material for artificial joints.

To improve the quality of interbody fusion material, we are now engaged in developing an MWCNT/PEEK composite. PEEK is a highly biocompatible material possessing excellent

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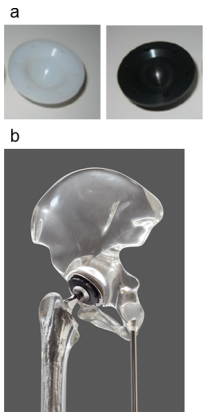

Figure 12. For application to sliding parts of artificial joints, an ultrahigh molecular weight polyethylene (UHMWPE) conjugated with MWCNTs has been developed. (a) A UHMWPE socket (left panel) and an MWCNT-conjugated UHMWP socket (right panel) for use in sliding parts of artificial joints. (b) A prototype artificial joint with a socket made of CNTs. Having favorable characteristics that have not been achieved with conventional materials, that is, high wear resistance and low breakability, MWCNT-conjugated UHMWPE is suitable as a sliding parts material for artificial joints.

biological safety and mechanical characteristics.[519,520] Because of its low compatibility for bone tissue, however, PEEK has been associated with the problem of insufficient bone union when used in implants that are directly exposed to bone, such as interbody fusion cages.[521−524] MWCNTs have been reported by many research teams, including ours, to possess bone induction potential.[58,62,63,67,213,215,216,218,525,526] If conjugation with MWCNTs further improves the mechanical characteristics of PEEK and also induces osteogenesis, then MWCNT/PEEK composite will become an ideal interbody fusion material (Figure 13).

In 2012, the European Commission (EC) announced a draft regulation as amended to oblige manufacturers of medical equipment used to make nanomaterial-containing products, to properly label medical devices containing nanomaterials categorized under Class III (most dangerous substances). This rule shall apply only in cases where such medical devices are used for the intended purposes and in the absence of measures (such as encapsulation and coupling) to prevent nanomaterials from entering the patient's body and the user's body.[527] Hence, use of CNTs composites as biomaterials may not be subject to legal regulations because they are bound to the base material. For this reason, we believe that stage 1 poses only a minimal safety risk and can be safely implemented, provided the appropriate legal procedures of each country are followed.

In stage 2, CNTs particles are used within the body. This stage represents the first high barrier to clinical application of CNTs because nanoparticulate substances come into direct contact with the body. This usage is subject to legal regulations according to the definition of the EC, and is thought to require international approval from an ethical viewpoint as well. Hence, research activities cannot proceed to this stage until an extensive assessment is performed following the establishment of international standards for evaluation of biosafety. Initially, the use of CNTs must be limited to localized sites. Furthermore, top priority should be given to the use of CNTs in situations where the benefits from their use by far outweigh the risks involved. Specifically, the most likely field

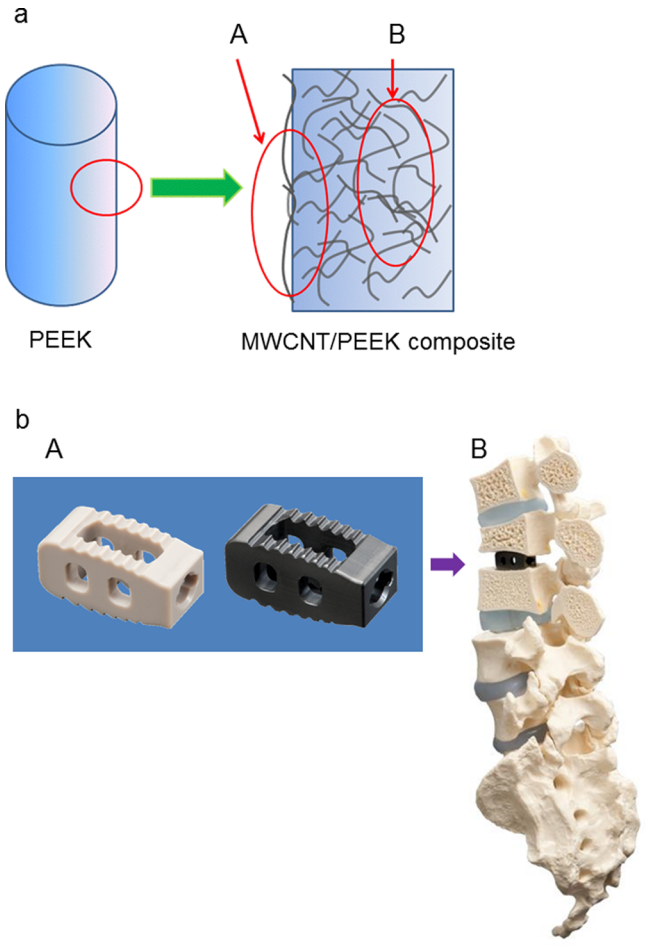

Figure 13. For application to spine interbody fusion material, a polyetheretherketone (PEEK) composite with MWCNTs has been developed. (a) A conceptual diagram showing that PEEK, when conjugated with MWCNTs, will become an innovative spine interbody fusion material possessing excellent mechanical characteristics and bone compatibility. (A) The MWCNTs on the surface confer bone compatibility. (B) The internally conjugated MWCNTs control the elastic modulus. (b) (A) A PEEK spine interbody fusion cage (left panel) and an MWCNT-conjugated PEEK cage (right panel). (B) A prototype interbody fusion cage made of CNTs.

appears to be cancer treatment, where no other treatment is available or treatment with CNTs is highly advantageous over other treatments. This is currently the most hopeful field of clinical application of CNTs. It is evident that if CNTs become applicable to DDSs and imaging for cancer treatment, dramatic advances in the treatment and diagnosis of cancer will be achieved, which is expected to contribute substantially to the health and welfare of many patients.

Stage 3 also concerns the topical use of CNTs particles as in stage 2, but the coverage is expanded to include the treatment of diseases requiring higher safety than in stage 2. CNTs are used clinically in topical treatments (including regenerative medicine scaffolds) and for the treatment and diagnosis of diseases that are less life-threatening than cancer, such as diabetes mellitus. In this stage, coverage of target diseases and use sites is much wider, and application of CNTs biomaterials more common. Stages 2 and 3 involve the same level of risk but have different benefits.

Finally, we will proceed to stage 4 aimed at the treatment of diseases involving the injection of CNTs and their systemic

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circulation via the bloodstream for the purpose of drug delivery and whole-body imaging. However, this decision requires extremely careful consideration.[327] As of 2012, the EC had approved 20 nanopharmaceuticals (of course, other than CNTs).[301] Although drug delivery and whole-body imaging using CNTs are highly effective procedures, major risk arises from their systemic circulation via the bloodstream. No clinical application should be started until the disposition of CNT particles and their effects on the heart, lung, liver, spleen, kidney, and other organs are extensively investigated and sufficient data are available to obtain an international consensus. At present, it remains unknown whether research activities will advance to stage 4.

It is important to make steady progress through these stages of clinical application and exercise discretion to demonstrate the safety of CNTs at each stage. This biological application and technical improvements in the biological application of CNTs would help accelerate the development of groundbreaking new therapeutic methods.

6.5. Establishing International Standards for Biological Safety Evaluation

To date, studies evaluating CNTs biomaterials safety have been conducted all over the world; however, interpretation of the collective results has been problematic because different methods of assessment were used by different researchers. Hence, it has been impossible to build a centralized toxicity database, which is essential for the assessment of CNTs safety and efficiency in biological systems.[91] International standards for biological safety evaluation need to be established as soon as possible, to conduct toxicity studies using one method of assessment and one set of standards, and to provide access to all results internationally. By doing so, many reliable results from all over the world can be analyzed by many experts, allowing them to make the right consensus decision. There are a great many types of CNTs and numerous derivatives produced by chemical modification. To achieve safe clinical application of these CNTs as soon as possible, there is an urgent need to establish international standards for the evaluation of biosafety.[70,191]

In the biological application of CNTs, it is critical to evaluate the safety of functionalized CNTs (f-CNTs), which are likely to find application as DDSs, for in vivo imaging, and in regenerative medicine scaffolds. Chemical modification is also important to increase the dispersion efficacy of CNTs, a key to successful biological application.[331] Of course, f-CNTs must be examined for safety individually. Furthermore, some researchers are working to functionalize CNTs to make them safer to living organisms.[257,334,528] To facilitate the application of numerous f-CNTs as biomaterials, it is of paramount importance to establish international standards for safety evaluation.

Provided that criteria are logically formulated on the basis of the published results from studies evaluating the safety of CNTs biomaterials, international standardization of the CNTs safety evaluation methodology would not be difficult. The first task is to establish standards for the topical use of CNTs. Specifically, in vivo and in vitro studies should first be conducted in the same manner as with ISO-standardized ordinary bulk biomaterials to assess the toxicity resulting from the dissolution of impurities contained in CNTs and some or all of the molecules bound to the CNTs. In vivo studies then should be conducted to assess the CNTs toxicity intrinsic to their identity as nanoparticles. This involves implantation of CNTs at the sites of their potential use to determine biocompatibility with a particular organ or tissue. The in vitro studies involve the dispersion of CNTs with a standard dispersant and use of ISO-compliant test methods similar to those used for ordinary chemical substances.[467] The in vivo and in vitro studies for determination of the intrinsic toxicity of CNTs involve comparison with a nanoparticulate reference material, carbon black as described above. With a standard reference, international standards for the evaluation of the biological safety of topically used CNTs particles can be established without delay.

Subsequently, efforts will be made to establish international standards for the evaluation of CNTs safety in applications involving passage through the bloodstream. Basically, in vivo studies on CNTs well dispersed in solution will be conducted using the same criteria as those used for ordinary chemical substances. However, it is unknown which substance (possibly an existing nanoparticulate material already used clinically in DDSs and possibly transported through the bloodstream, with confirmed safety and properties similar to those of CNTs) will be the appropriate reference material. Selection of a reference for this application of CNTs, which circulate in the bloodstream, is a major challenge to be tackled in the future.

In all cases, international standards for the evaluation of CNTs biosafety need to be established as soon as possible because ultimately CNTs will revolutionize cancer treatment and regenerative medicine, which are top priorities in today's medicine. Now is the time to translate research on safe CNT composite implants into clinical applications. International standards for evaluation of CNTs biosafety must be established to enable the topical use of CNTs particles. Research into any important medical issue should always proceed without interruption.

7. CONCLUSION

The study of the application of CNTs as biomaterials has been increasing dramatically because CNTs have been shown to be extremely effective and very safe biomaterials. Biomaterials that have doubtful biosafety are unlikely to find clinical application in the future. Although it is logically impossible to say that CNTs are completely safe to use in living organisms, CNTs can be judged to be extremely safe if no evidence of biological risk has been obtained by a vast number of studies investigating their biological application. Most researchers in this field think CNTs are safe to use in living organisms, provided that the appropriate method and site of delivery are used.

CNTs biomaterials if fully utilized could lead to many revolutionary and important medical technologies. Because of the extremely advantageous characteristics unique to CNTs, the biological safety evaluation issue making us reluctant to start their clinical application must be solved as soon as possible.

Thanks to the painstaking efforts of a great many researchers, much evidence supports the claim that CNTs are generally safe as biomaterials. Accordingly, now is the time to start clinical application of CNT composite implants, the biologically safest form of CNTs, because there is little possibility that CNTs will be directly exposed to the living organism. To quickly proceed topical use of CNTs particles, it is necessary for researchers to establish international standards for biosafety evaluation as soon as possible. In this process, the carbon black reference will play an important role. When taking the next and most risky step toward clinical application (that involves the entry of

CNTs into the circulation), the utmost caution must be exercised to ensure safe use.

Because many researchers can now evaluate the biosafety of CNTs using the power of the latest science and technology, we should now embark on a journey toward the clinical use of CNT-based biomaterials in an ethical and courageous manner.

AUTHOR INFORMATION

Corresponding Author

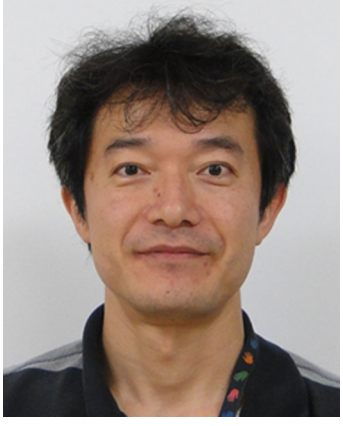
*Tel.: +81 263 37 2409. Fax: +81 263 35 8844. E-mail: saitoko@shinshu-u.ac.jp.

Notes

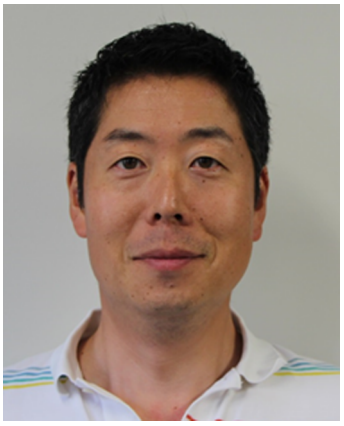
The authors declare the following competing financial interest(s): As an employee of a medical device development company, Naoyuki Nishimura may benefit financially from participation in this study in the future. No other authors have conflicts of interest.

Biographies

Naoto Saito is a professor and director of the Institute for Biomedical Sciences, Shinshu University. He is an experienced researcher specializing in biochemistry, cell biology, regenerative medicine, biomaterials, and nanobiotechnology. As the leader of Shinshu University's Nanobiotechnology and Biomedical Engineering Team, he is working on developing CNT-based biomaterials.



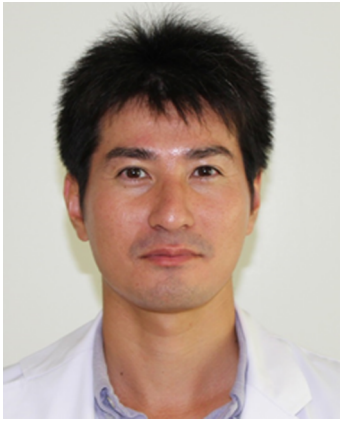
Hisao Haniu is serving as a lecturer in the Department of Orthopaedic Surgery, Shinshu University School of Medicine. He is a qualified clinical laboratory technician and specializes in physiology and biochemistry, especially in proteomics. He is engaged in research on the safety evaluation of nanomaterials and in the training of many young researchers.



Yuki Usui is an associate professor at the Research Center for Exotic Nanocarbons, Shinshu University. He is an orthopedist, specializing in research on the biological application of nanomaterials. He showed for the first time that CNTs promote bone formation in an animal study on bone tissue regeneration.

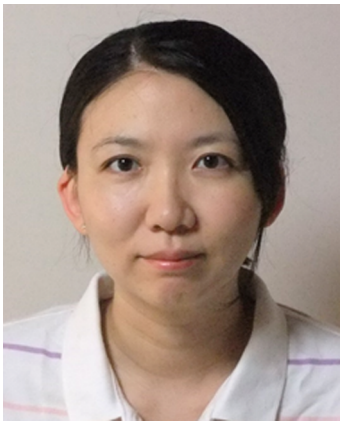
Kaoru Aoki is serving as a research associate in the Department of Orthopaedic Surgery, Shinshu University School of Medicine. He is a medical doctor specializing in bone and soft tissue tumors, and also a rehabilitation doctor. He is concurrently engaged in basic research and clinical research, conducting research to apply nanocarbons as drug delivery systems for anticancer agents.

Kazuo Hara is an orthopaedic surgeon in the Department of Orthopaedic Surgery, Shinshu University School of Medicine. His special interest is evaluation of the biological safety of CNTs as biomaterials.



Seiji Takanashi is a researcher at the Shinshu University School of Medicine, and is also serving as a medical doctor in the Department of Orthopaedic Surgery. He is working on evaluating the carcinogenicity of CNTs in transgenic mice and clarifying the action of nanomaterials in cells.

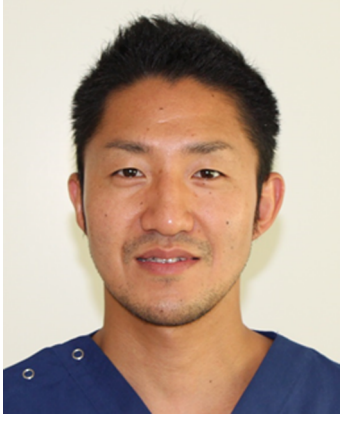
Masayuki Shimizu serves as a research associate in the Department of Orthopaedic Surgery, Shinshu University School of Medicine. He is a medical doctor specializing in spinal surgery, and conducts both basic and clinical research. He is exploring the mechanism of bone formation promotion by CNTs at the cellular level.



Nobuyo Narita is a research associate in the Department of Orthopaedic Surgery, Shinshu University School of Medicine. She specializes in lower limb surgery, serving as a leader of the foot surgery team at Shinshu University Hospital. She demonstrated using intracellular signals that CNTs suppress the function of osteoclasts.

Masanori Okamoto is a researcher at the Shinshu University School of Medicine, and is a medical doctor in the Department of Orthopaedic Surgery. He is now studying bone metabolism at Matsumoto Dental University and has a special interest in the action of Wnt5 in osteoblasts.

Shinsuke Kobayashi is a researcher at the Shinshu University School of Medicine. He specializes in safety evaluation of nanomaterials, and is investigating the in vivo kinetics of CNTs using MRI and CT.



Hiroki Nomura is a researcher at the Shinshu University School of Medicine. As a member of the research and development team for application of CNT-conjugated polyethylene to artificial joints, he is engaged in evaluating the biological safety of CNTs and their composites.

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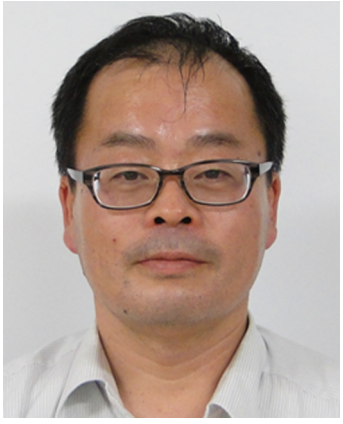
Hiroyuki Kato is a professor in the Department of Orthopaedic Surgery, Shinshu University School of Medicine. He specializes in hand surgery and conducts research on nerves and tendons. He provides research and clinical practice training to many physicians in the Department of Orthopaedics at Shinshu University Hospital.



Naoyuki Nishimura is the director of the R&D Center, Nakashima Medical Co. Ltd. He is developing artificial joints and spinal fixation devices made of CNT composites jointly with Shinshu University. He investigates safety and regulatory science with the aim of clinical application of CNT composites.

Seiichi Taruta is a professor in the Faculty of Engineering, Shinshu University. He is working on developing CNT-conjugate ceramics and bioactive ceramics. He is also a member of a medicine-engineering collaboration research project to develop CNT-based artificial joints.

Morinobu Endo is a distinguished professor in the Faculty of Engineering, Shinshu University. As the discoverer of the manufacturing process for CNTs using a chemical vapor deposition approach, he has been a world leader in CNTs research. As head of the Research Center for Exotic Nanocarbons, he currently conducts research on multitargeted application of nanocarbons.

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ABBREVIATIONS

AFP	α-fetoprotein
AIST	National Institute of Advanced Industrial Science and Technology
ALP	alkaline phosphatase
APC	antigen-presenting cell
bmDC	bone marrow-derived dendritic cell
CA19-9	carbohydrate antigen 19-9
CEA	carcinoembryonic antigen
CNS	central nervous system
CNT	carbon nanotube
CVD	chemical vapor deposition
DDS	drug delivery system
DTPA	diethylenetriaminepentaacetic acid
DTPA	diethylenetriaminepentaacetic acid
EDS	energy dispersive X-ray spectroscopy
ELISA	enzyme-linked immunosorbent assay
ES cell	embryonic stem cell
f-CNT	functionalized-CNT
GEM	gemcitabine
IC_{50}	half maximal inhibitory concentration

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IL	interleukin
iPS cell	induced pluripotent stem cell
ISO	International Standards Organization
LPS	lipopolysaccharide
MNU	*N*-methyl-*N*-nitrosourea
MWCNT	multiwalled CNT
NIOSH	National Institute for Occupational Safety and Health
NIST	National Institute of Standards and Technology
OECD	Organization for Economic Co-operation and Development
PEEK	polyether ether ketone
PEG	polyethylene glycol
PSA	prostate specific antigen
rhBMP-2	recombinant bone morphogenetic protein-2
ROS	reactive oxygen species
SEM	scanning electron microscopy
siRNA	short interference RNA
SWCNT	one layer is known as single-walled CNT
TCB-1	Tattoo carbon black-1
TCB-2	Tattoo carbon black-2
TEM	transmission electron microscopy
TNF	tumor necrosis factor
UHMWPE	ultrahigh molecular weight polyethylene
ZDBC	zinc dibutyldithiocarbamate

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