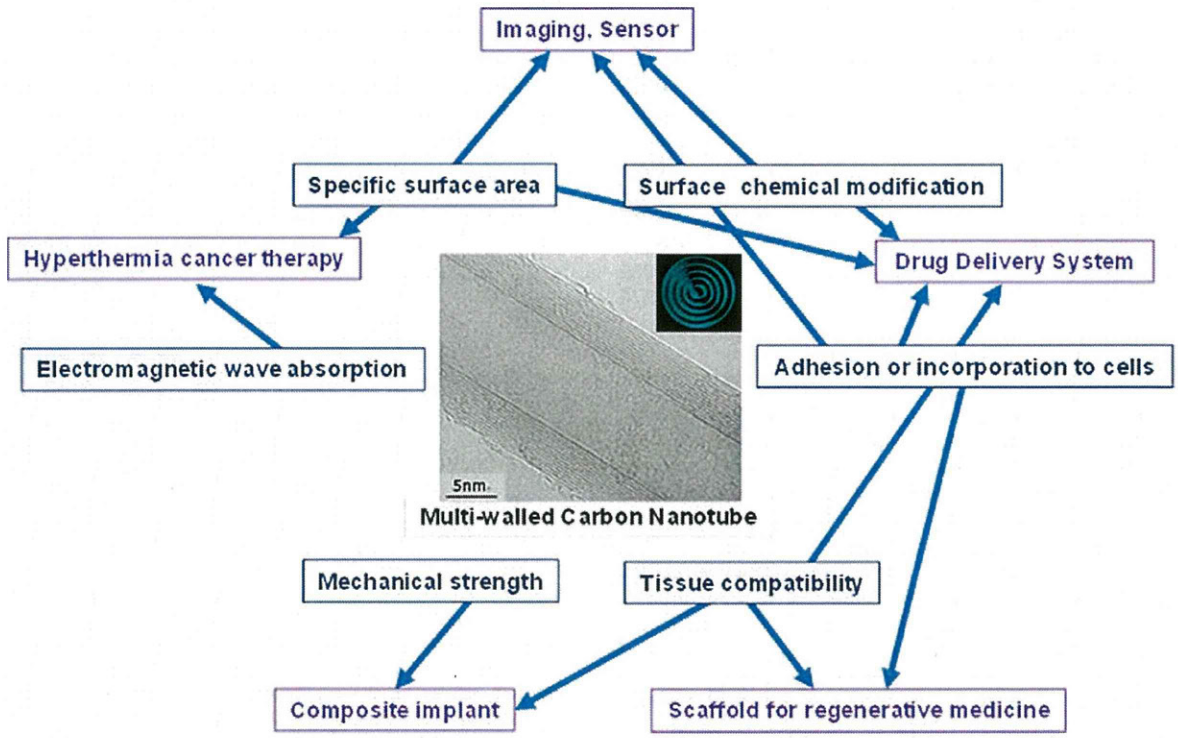

Figure 2. Biological applications of CNTs encompass a broad range of fields, many of which, in addition, represent themes of top priority in today's clinical medicine, such as cancer treatment and regenerative medicine. Modified from ref 84, which is published under the Creative Commons Attribution License.

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

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

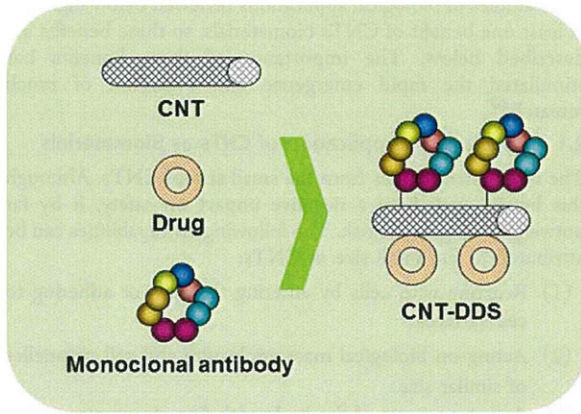

Figure 3. CNTs are capable of working as a platform for concurrently binding drugs such as anticancer agents, proteins, and peptides such as monoclonal antibodies, high molecular polymers, and other molecules that otherwise cannot be bound to each other. Making the best use of this feature, it would 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

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]

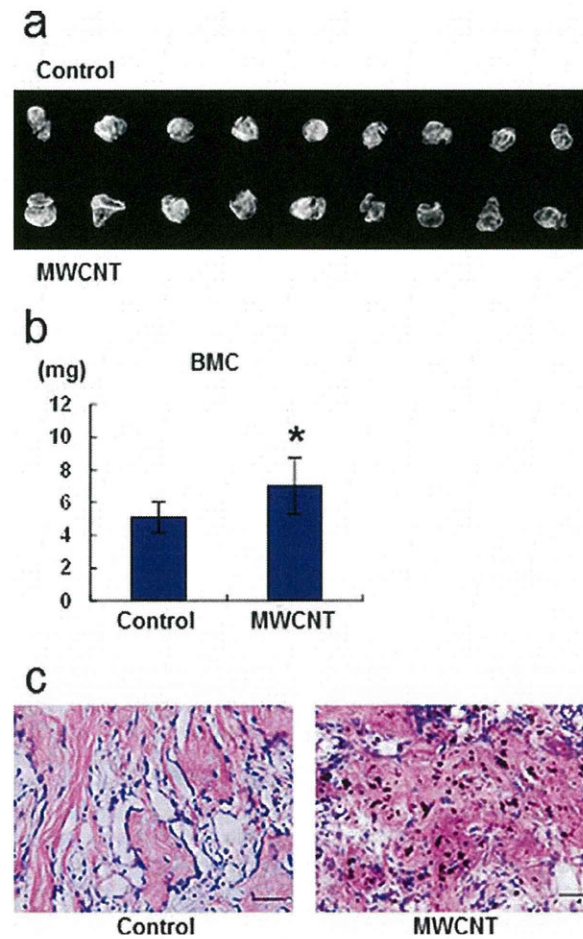

Figure 4. MWCNTs promote ectopic osteogenesis by rhBMP-2 and collagen. (a) A soft X-ray radiogram of newly formed bones extirpated 2 weeks after placement of rhBMP-2/collagen/MWCNT composite (upper lane) or rhBMP-2/collagen composite (lower lane) in mouse back muscle. Larger bones with more intense opacity 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

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

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.

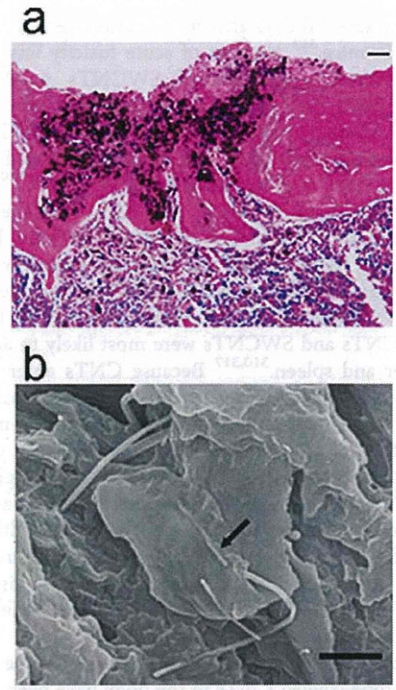

Figure 5. MWCNTs exhibiting good bone compatibility as they are absorbed in repaired bone without interfering with bone repair. (a) A histological image of a tibia extirpated 4 weeks after surgery for implant of MWCNTs in a pit drilled in tibial diaphysis after incising the anterior surface of a mouse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

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.