Title

Bioactive Nanocarbon Assemblies: Nanoarchitectonics and Applications

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Abstract

Graphene, carbon nanotubes, and fullerene are representative nanocarbons which have zero, one, or two dimensional structures, respectively. These nanocarbons can be used as building blocks for construction of higher dimensional or complex materials by nanoarchitectonics; a technology used to control nanoscale structures and spaces. By combination with other materials and/or devices, nanoarchitectures of nanocarbons can be formed into structures of different dimensions and properties for biological

applications. In this review, biological applications, especially cell growth, sensing, and control **using** nanoarchitectures of nanocarbons are summarized.

Main text

Introduction

Nanocarbons such as graphene, carbon nanotubes (CNTs) and fullerene have unique properties as electronic materials, and are indispensable in the field of **nanotechnology**. Nanotechnology is one of the most important keywords in current science and technology. It allows control over nano-sized and/or near-nano-sized structures and materials for the creation of novel functionality with excellent efficiency and specificity [1; 2]. **In this respect, and as Richard Feynman claimed,** there's plenty of room at the bottom [3]. Nanotechnology presents many possibilities often based **on methods** involving so-called top-down approaches in which microscopic and nanoscopic systems are fabricated from bulk materials using sophisticated techniques. However, there exist intrinsic limitations of the current technologies which are expected to be reached in the near future. Therefore, we would like to establish an alternate approach, the so-called bottom-up approach, to construct functional structures from atoms and molecules **by using** spontaneous phenomena such as self-assembly and supramolecular processes [4-7], as well as controlled processes such **as film preparation using Langmuir-Blodgett (LB) techniques** [8; 9] or layer-by-layer (LbL) assembly [10-12]. Although these approaches can be regarded as modern concepts, biological systems have been **evolved** based on self-assembly processes involving construction of finely tuned functional **structures**.

“Nanotechnology” in a biological context differs from man-made

nanotechnology in that nanometric biochemical functions involve massively parallel and highly integrated processes operating extremely sophisticated mechanisms. The novel concept of “nanoarchitectonics” has been recently proposed by Aono [13]. “Nanoarchitectonics” is a technology system aimed at arranging nanoscale structural units—a group of atoms, molecules, or nanoscale functional components—into a configuration that creates a novel functionality through mutual interactions among those units [14]. This concept aims to produce new functionality in whole assembled units through concerted interactions within nanostructures [15-24] by analogy with those found in living systems.

In order to gain a fundamental understanding of and further promote nanoarchitectonics, we should apply key components for preparation of the resulting unique architectures. Ideally, these components should be composed of very simple elements of well-defined dimensions. There exists a family of nanomaterials that satisfies these conditions: the family of nanocarbons [25-27]. Zero-dimensional (0D) fullerenes, one-dimensional (1D) carbon nanotubes (CNTs), and two-dimensional (2D) graphene are included in this category of materials. In addition, all these substances are composed of carbon, which is also the most common element in biological materials “selected” by nature for its diverse and flexible chemistry. Therefore, approaches to biological systems using nanocarbon materials based on the nanoarchitectonics concept makes an interesting analogy and may present unexpected opportunities for technological progress. Although there exist some concerns over the toxicity of nanoparticles [28], cytotoxicities of nanocarbons are thought to be moderate to none according to the recent research [29; 30]. Against this background, we have here reviewed recent examples of bioactive nanocarbons involving nanoarchitectonics and

applications according to their dimensional classification: lower dimensional nanocarbons are used (or combined with other materials) to construct higher dimensional and multi-functional materials by the application of recent technologies. The concept of this review is summarized in Fig. 1. It is focused on recent developments in nanoarchitectonics of nanocarbons that can sense and control cell-activities. Nanocarbons for tissue engineering [31; 32], applications of nanocarbons to medicine [33; 34], toxicity of nanocarbons [29], or nanotopography-induced cell control for which non-nanocarbon materials have been used [35] were summarized in previous reports, and they are not introduced in detail in this review.

1. Two-dimensional nanocarbon: Graphene

Graphene has a 2D structure consisting of an sp^2 carbon honeycomb-network [36-40]. Graphene, graphene oxide (GO) and reduced graphene oxide (rGO) make up the family of graphenes. Since its first isolation by mechanical exfoliation of graphite crystals using Scotch® tape, a variety of its tremendous properties have been discovered. Graphene is mechanically strong, lightweight, and nearly transparent, as well as being an excellent conductor of heat and electricity; features which originate from the fact that graphene consists entirely of sp^2 carbons. For biological applications, the extreme hydrophobicity and excellent potential for adsorption of hydrophobic molecules should be noted [41]. Various methods for preparation of graphene have been reported including mechanical exfoliation of graphite, chemical vapor deposition (CVD), chemical and mechanical cutting of carbon nanotubes, etc. Of these, CVD is preferred due to its potential for large scale synthesis of high quality graphene. GO has a 2D structure, which consists of an sp^2 and sp^3 carbon network but includes oxygen (and

hydrogen), and has defects in honeycomb structure. It is generally synthesized by chemical oxidation of graphite leading to graphite oxide (GO) that can be exfoliated. Due to the introduction of oxygen-containing functionalities, it gains some hydrophilicity, and has a rather flexible, soft structure. Although its electronic properties are inferior to those of graphene, application of GO in biological systems has several advantages due to its good processability. Since it is soluble in several solvents, simple transfer processes such as spin-coating, electrospraying, compressing, filtration, LbL techniques, etc. can be adapted. rGO has a 2D structure consisting mostly of sp^2 carbon. It is synthesized by chemical reduction of GO [42]. Although its detailed structure is still under debate, it is considered to have a honeycomb-network of sp^2 carbon containing a variable number of defects. Due to the strong π - π interactions between graphene sheets, it is difficult to obtain processable pristine graphene sheets in large quantities. rGO is considered to be a single type of chemically derived graphene, which can be obtained as single sheets. Although full reduction of GO to graphene remains difficult, the conductivity of rGO is 4 orders of magnitude higher than GO [43]. Although graphene/GO/rGO have 2D-structures, various techniques can be used to form them into 3D-structures. In the following section, multi-dimensional structures as cell culture platforms are summarized.

1.1 Two-dimensional nanoarchitectures of graphene for cell growth

The surfaces of graphene and its derivatives have been used as platforms for cell culture. Although graphene and rGO are highly hydrophobic, several reports have shown that this environment may be suitable for cell culture. On the other hand, the surface of GO contains functional groups that can be used for chemical reactions

leading to covalent functionalization of the GO sheet. GO sheet can also be functionalized by utilizing non-covalent interactions. Graphene and GO have comparable effects on attachment and proliferation of cells but differ in their effects on cell differentiation. When mouse myoblast C2C12 cells were cultured on 2D thin films of GO, rGO or on glass, the remarkable differentiation ability of GO could be observed, and was monitored by expression of specific genes (MyoD, myogen Troponin T, and MHC) [44]. There have been conflicting reports on the effect of graphene/GO/rGO on cell behavior, which might originate from differences in the cell-types used or from the different methods used to obtain graphene/GO/rGO (so that microscopic structures and purities could also be subject to variation). In one report, 25 µg/ml GO gave a nonspecific enhancement of cell growth both in bacterial culture (*E. Coli*) and mammalian cell cultures (human adenocarcinoma HT-29 vrlld) [45]. This conflicts with previous results which indicated that GO has antibacterial properties [46; 47]. However, since GO had been purified more thoroughly in the later reported case, the authors of reference [45] proposed that impurities introduced during preparation of GO could have led to the previously observed antibacterial effect reported in references [46; 47]. Cell growth on graphene was promoted compared to SiO₂ substrates when human osteoblasts and mesenchymal stem cells (MSCs) were used [48].

Since the structure and properties of GO vary according to the degree of reduction, fine tuning of the reduction procedure is necessary for controlled cell adhesion. Few-layer reduced GO films were prepared by low-temperature thermal reduction of GO [49]. By varying the reaction time and temperature, the state of reduction (i.e., the oxygen content) can be controlled. Although there was no significant morphological difference between graphene samples, cell attachment was dramatically

altered. The film with moderate reduction level allowed better cell attachment and proliferation compared to unreduced or over-reduced films. Apart from the reaction time and temperature, the reagent can also affect the state of reduction. A self-supporting graphene hydrogel was provided by chemical reduction of GO using hydrazine and NH_3 , which resulted in dispersion of reduced GO that spontaneously gels at the solid-liquid interface during filtration (Fig. 2) [50]. The resulting sheets are stable in the absence of detergents and were used as a platform for bone regeneration. The film was found to stimulate osteogenic differentiation of stem cells without addition of inducers, both in vitro and in vivo. It was also found from in vitro study using rat bone marrow stromal stem cells that osteogenic differentiation and calcium deposits of stem cells occurred. Wrinkles of graphene can strongly adsorb proteins and enhance cell growth and differentiation. In the in vivo study of implantation to subcutaneous dorsum sites of rats, implantation was successful without severe inflammatory response. Formation of a thin layer fibrous capsule and a new blood vessel was observed suggesting cell differentiation.

Patterned functionalized graphene can be obtained by printing. The surfaces of non-conducting graphene derivatives were patterned. When graphene was treated with XeF_2 , fluorinated graphene whose aromaticity had been removed was obtained (Fig. 3) [51]. Fully fluorinated graphene showed higher proliferation over partially fluorinated graphene. When fully fluorinated graphene was patterned by printing polydimethylsiloxane (PDMS) as a blocking agent, MSC cells aligned only on the fluorinated graphene. The morphologies of the cells became narrow with growth along the exposed fluorinated graphene. In the absence of inducing agent, a stronger differentiation effect was observed for patterned fluorinated graphene over an

un-patterned one.

The origin of different cell behavior such as cell growth and differentiation has been investigated using stem cells (Fig. 4) [52]. It has previously been reported that graphene can promote adhesion and proliferation of MSCs and differentiation of MSC in the presence of certain chemicals, although the reason was unclear. Those authors found that the key properties are **adsorption** and denaturation of cell regulating molecules and proteins by π - π , **electrostatic, and hydrogen bonding interactions mediated by GO or graphene film**. The authors prepared multilayer-GO sheet by using the LbL method. Thus, PMMA-coated graphene was transferred onto the first layer graphene on copper foil. After etching the copper foil, the two-layer graphene film was transferred onto a third layer graphene on copper foil. Based on these steps, **multilayer-graphene was transferred to substrates, followed by the removal of the top PMMA**. The authors prepared multilayer-graphene film through the LB method. Thus, **GO was dispersed in water-methanol solution, and the GO film was formed by dip-coating the substrate several times**. Graphene can **adsorb** and accumulate osteogenic inducers (= dexamethasone and β -glycerol phosphate), and promote osteogenic differentiation. The time **required** to induce osteogenic differentiation was **reduced** (from 21 to 12 days) compared with differentiation on polystyrene with inducers. Graphene adsorbed and denatured insulin, a key regulator for the synthesis of fatty acids. As a result, graphene suppresses adipogenesis. On the other hand, GO (which has hydrophilic **functional groups on its surfaces**) can **adsorb** insulin by electrostatic interactions but does not denature it. Strong π - π **interactions** between graphene and proteins **cause** their denaturation as illustrated by the case of insulin.

Recently, graphene was reported to be a good platform for gene delivery in

induced pluripotent stem cells [53]. Transfection efficiency using baculovirus was similar between the cells cultured on glass, graphene, and GO, although it was reported to be increased on graphene using NIH-3T3 cells. Size-dependent cell uptake of protein-coated GO has been reported [54]. GO was labelled using fluorescein modified bovine serum albumin and separated by centrifugation. Large (5 μm) and small (1 μm) particles of protein-coated GO were placed on C2C12 cells and time-dependent cellular uptake was observed. It was found that these particles were internalized to cells by endocytosis, which is a similar mechanism of internalization as for other nanoparticles.

1.2 Three-dimensional nanoarchitectures of graphene for cell growth

Since most cells in tissue exist within a three-dimensional (3D) scaffold, cell cultures on 3D architectures might be a promising method for tissue engineering. 3D nanoarchitectures composed of 2D graphene can be obtained by coating graphene onto 3D nano-scaffolds. Nano-fibrous scaffolds can be fabricated by electrospinning of polymers with subsequent deposition of graphene by **using** the LbL method (Fig. 5) [55]: **A 3D scaffold of poly- ϵ -caprolactone nanofibers was activated by soaking it in polyethyleneimine (PEI) solution buffer then dipping in heparin-graphene solution, followed by being soaked in poly(L-lysine) (PLL) solution. This LbL assembly deposition cycle was repeated so that a polymer-based graphene 3D scaffold was fabricated.** LbL assembly is characterized by a steady mass increase during an in situ deposition process in 2D. Neural cells, day 5 primary cortical neurons, were cultured on this polymer-based graphene 3D scaffold. There was no difference in cell toxicity and morphology between the graphene 3D scaffold and the non-graphene-coated scaffolds. These results **indicate** that this graphene 3D scaffold is a biocompatible material. 3D

scaffold of graphene **was** also obtained by deposition of graphene sheet onto a substrate that has a 3D structure (Fig 6) [56]. A large graphene sheet, with dimensions of 1 x 1 cm, was synthesized by the CVD method on copper foils and was then transferred to four substrates, Si/SiO₂, glass, polyethylene terephthalate (PET) film, and PDMS. AFM observations revealed that the graphene surface of each material had different ripples and wrinkles at the nanometer scale. Graphene on glass or Si/SiO₂ showed good compatibility with human MSCs (hMSCs) while graphene on PET or PDMS exhibited lower cell viability. From the control experiments, cell toxicity was revealed to originate from the 3D structure of the mother substrates themselves. On the other hand, differentiation properties were not affected by the 3D scaffolds. Three of the **substrate** combinations, graphene on Si/SiO₂, glass or PET film, were tested for use **in** cell differentiation. Differentiation of cells was monitored using typical markers: CD-44 for hMSCs and osteocalcin for osteoblasts. Presence of graphene on the substrates accelerated cell differentiation even in the absence of commonly used additional growth factors such as BMP-2. In this case, graphene was the driving force for bone cell formation, regardless of the underlying substrate.

1.3 Sensing and control of cell functions on graphene nanoarchitectures

Graphene is a material which possesses high electrical conductivity and can thus be applied as an active material in devices for sensing of cell functions. Graphene can be used as a non-contact electrode [57]. An electrical field was formed between two graphene electrodes and neural cells (SHSY5Y human neuroblastoma cells) were cultured between the electrodes covered with PET sheet. Neural cell-to-cell interactions could be controlled by applying an electrical stimulus. Patterned rGO films have also

been used as field effect transistor (FET) devices, and were synthesized by the micro-molding-in-capillary (MIMIC) method (Fig. 7) [58]. The pattern was obtained by using a stamp of grooved PDMS on a flat PET film. A solution of GO was dropped in the grooves and dried. After removing the PDMS stamp, the resulting patterned GO was chemically reduced to patterned rGO. The resulting patterned rGO film was used as conducting channel and the FET device prepared using this rGO film could be used to detect dopamine since strong π - π interactions between the aromatic moiety of dopamine and graphene affected the Dirac point in the I-V curve of the device. Release of catecholamines from individual P21 cells on this film could be detected by observation of current spikes.

Because of its large contact surface area, high electric conductivity and wide electrochemical window, graphene is an ideal material for electrochemical sensing. Real-time detection of nitric oxide (NO) was achieved by culturing cells on peptide-immobilized graphene sheet [59]. NO can be detected using graphene since it is oxidized on contact with graphene and so can be monitored by observing the current variation. On the other hand, detection of low concentrations of NO released from cells is difficult. The most effective way to detect NO is to place a sensor in close proximity to cells. This can be achieved by growing cells on the sensing element, in this case a graphene sheet. Since strong attachment of cells on graphene is critical for detection of NO, covalent attachment of arginine-glycine-aspartic acid (RGD)-peptide (which promotes cell adhesion) at the graphene sheet leads to high sensitivity and good selectivity of NO detection. Previously available electronically-active nanomaterials mostly possess 0D (i.e., fullerenes) or 1D (i.e., carbon nanotubes) structures, and they have been used to detect biomolecules such as proteins, ATP, DNA or viruses. They can

also be used for interaction with nano-scale materials but they are not suitable for contacting micrometer sized materials such as cells. Thus, a 2D structure is beneficial for detection of molecules interacting with or emanating from cells. Graphene is an ideal material for in situ electronic cell assays due to its high conductivity and excellent cell adhesion properties [60]. Graphene has also been used for detection of hydrogen peroxide (H_2O_2), another of the reactive oxygen species (ROS) family (which includes NO), by a similar method. H_2O_2 also operates as a chemical messenger within cells. Although monitoring cellular release of H_2O_2 would be informative, quantitative detection of H_2O_2 released from cells is difficult due to the short half-life of H_2O_2 and the large radius of human (or other) cells. Thus, detection of H_2O_2 very close to the cells' surfaces is optimal and can be achieved by using graphene, a material that has good cell adhesion properties and conductivity. For efficient detection of H_2O_2 , the surface of graphene was modified and a layered film of graphene/artificial peroxidase/extracellular matrix protein was fabricated. This composite layered film enabled in situ selective and quantitative detection of extracellular H_2O_2 released from cells.

Graphene is an excellent material for absorption of near infrared (NIR) light-, and as a result, it can be used to generate local heating. By using this characteristic of graphene, a NIR-responsive cell adhesion system was developed [61]. In this system, dissociation of double-stranded DNA used as a linker between graphene sheet and cells was achieved by NIR-irradiation-induced heating. A combination of rGO, polymer, DNA-cell adhesive protein (RGD), and gold nanoparticles enabled fine tuning of photothermo-activity of the materials and biocompatibility. Thus, cells could be attached to a graphene surface through RGD, and a linker of double stranded DNA. Irradiation with light of NIR wavelength, caused heating so that double stranded DNA

was dissociated. This result indicates that graphene can be an active platform for the control of cell attachment.

Graphene can also be used to effectively quench fluorescence by electron or energy transfer. This phenomenon can then be used for detection of metal ions by using a highly aromatic ion sensor [62]. An amino-functionalized pyrene was used to bind metal ions, and the resulting complex interacted with graphene through π - π interactions. Quenching of fluorescence induced by photoinduced electron transfer was blocked when the sensor molecule was bound to Mn^{2+} . When Hela cells were cultured on this graphene nanosheet-pyrene composite, it was taken up by cells and internalization of Mn^{2+} by the cells could be monitored.

Graphene has remarkable electronic properties and can be used to detect attachment of individual molecules to cells on its surface. On the other hand, the selectivity of graphene itself for particular molecules or cells is limited. Selectivity can be improved by combining graphene with other materials that have appropriate selective affinities for the required molecules or materials. Graphene has been applied, together with a material that has high affinity for cancer cells, in an electrochemical cytosensor that can be used to detect tumor cells with high selectivity [63]. In this case, folic acid was used to selectively attach the tumor cells human leukemic cells (HL-60) or human lung cancer cells (A549). First, a chitosan-graphene hybrid material was prepared by reduction of GO in the presence of a chitosan derivative. In a second step, functionalization of this chitosan-graphene material was achieved by LbL assembly of PEI and folic acid. This resulting hybrid material of folic-acid-chitosan-graphene was used to detect the attachment of tumor cells by means of fluorescence or electrochemical signals.

2. Carbon Nanotubes

Carbon nanotubes (CNTs) have 1D structures consisting of single- or multi-layered graphene sheets rolled up in the form of cylinders [64-67]. The former are known as single-walled carbon nanotubes (SWCNTs), and the latter multi-walled carbon nanotubes (MWCNTs). The electronic properties of CNTs such as whether they are semi-conducting or metallic is determined by how the 1D graphene is “wrapped”, or in other words, by their chirality. Thus, their structure is important for applications as materials for electronic devices. On the other hand, it is currently not practical to separate or synthesize selectively CNTs depending on their chirality. Furthermore, CNTs are obtained as a mixture of isomers having different radii, lengths, and chirality. CNTs are also prone to suffer from contamination by metal catalysts used in their synthesis, which are difficult to remove. Thus, for biological application of CNTs, one should be vigilant about their purity [68]. Various techniques have been developed for the synthesis of CNTs including arc discharge, laser ablation, and the CVD method. Similarly to graphene, CVD is one of the best methods for their cheap, large-scale synthesis. Also similarly to graphene, CNTs have remarkable properties such as high mechanical strength, excellent conducting properties of heat and/or electricity, and strong light absorption over a wide range of wavelengths. They differ distinctly from graphene in their high aspect ratios and relatively small 1-100 nm diameters, making them attractive for fabrication of assembled nanoarchitectures. CNTs have inner cavities where various compounds can be internalized [69; 70]. Metals, inorganic salts, and organic molecules have been reported to have been internalized into the inner cavities those where fullerene-C₆₀ was encapsulated are known as 'peapods'. Here,

multi-dimensional structures as platforms for cell cultures and cellular control are described.

2.1 Two-dimensional nanoarchitectures from carbon nanotubes for cell growth

Biocompatibility of CNTs with cardiac muscle cells has been tested revealing the lack of any observable cytotoxicity [71]. Uptake of CNTs was revealed to occur by a similar mechanism to that of nanoparticles [72]. Oxidized CNTs were encapsulated by RNA and the complex was internalized to cells by an endocytosis pathway.

Micro- and nanoscale- topography of organic and inorganic materials has been reported to influence cell functions such as cell growth, proliferation, differentiation, etc. Since graphene and CNTs have differing morphologies originating from their respective structures, these two nanocarbons can yield different biological effects. Viability of neuroendocrine PC12 cells was compared for films composed of SWCNT or rGO (Fig. 8) [73]. A film of SWCNT was obtained by phase-separation-facilitated self-assembly of dispersed SWCNTs. Since CNTs and graphene are highly hydrophobic and are difficult to handle in the solution phase, their respective oxidized forms, SWCNT-COOH and GO, that contain carboxylic acid groups and can be dissolved or dispersed in solvents, were used as starting materials. A thin film of SWCNT-COOH was obtained by phase separation and was transferred onto a glass coverslip. The film was then heated to remove the -COOH groups from SWCNTs forming a film of SWCNT. A film of rGO was obtained by spin-coating of redispersed GO onto Si/SiO₂ wafer, followed by reduction with hydrazine. From SEM and AFM analysis, SWCNT bundles were observed to form a nano-mesh structure with a roughness of 10 nm, while rGO film was almost flat with a roughness of 1 nm. Cells on the SWCNTs film showed

lower proliferation, viability, and differentiation ability compared with those on rGO. This result indicates that nano-sized wrinkles have the effect of depressing cell proliferation. The effects of graphene, MWCNTs and their hybrids on cell behavior were also systematically surveyed.

Due to their characteristic 1D structures, when synthesized by CVD methods CNTs can be grown **perpendicular** to the plane of the metal catalyst in a densely packed form known as “CNT forest”. A variety of 2D and 3D nanoarchitectures can be formed from this forest. **Transparent sheets of MWCNTs of 5-centimeters width and meters in length were formed by hand drawing of MWCNT forest (Fig. 9) [74; 75]. Drawing was initiated using an adhesive strip to contact MWCNTs and the sheet was extended by hand drawing. By this simple method, oriented transparent sheet of MWCNTs was fabricated. By overlaying these sheets in certain orientations, a sheet with a 2D reinforced structure was fabricated.** CHO cells were cultured on two different **reinforced** sheets made of this MWCNT **sheet** with **CNTs** aligned **parallel** in one and **arranged perpendicularly in another**. These MWCNT sheets were applied to a cover glass. When cells were cultured it was found that cells aligned along the MWCNTs bundles. **When aligned MWCNTs were used reduced clustering of cells was observed. Moreover, 16% of the cells had an aspect ratio of 3:1 or higher.** This result indicates that **CNTs** favor cell adhesion and can act as a platform for cell growth. Nanostructuring of CNTs has a large effect on cell adhesion, proliferation, and cytoskeletal development. **Sheets of CNTs** exhibit good **properties of** adhesion and proliferation compared with PET films, carbon fibers, or glass substrates [76]. **Sheets of CNTs** may bear similarities with natural extracellular **matrices**, which **promote** cell growth.

Carbon nanohorns (CNHs) are a family of CNTs with conical caps at the end of

their structures. CNHs not only have similar structures to CNTs but also have similar biological effects. Human osteoblast-like MG 63 cells were cultured on polysulfone wrapped CNHs or CNTs with both exhibiting similar properties of cell adhesion, spreading, and viability [77].

Hybrid materials of graphene and MWCNTs are also available for cell culture [78]. Graphene and MWCNT–graphene hybrid films were synthesized by CVD. Delicate human embryonic stem cells (hESCs) were cultured on MWCNT–graphene hybrid. hESCs were cultured on MWCNT–graphene hybrid with/without Geltex® and compared with that on graphene, SiO₂/Si, and glass. Geltex® is a matrix commonly used for cell culture of hESCs and is necessary for adhesion of hESCs. No difference was found in cell viability, pluripotentiality and proliferation. These results show that MWCNT–graphene hybrid is a promising material for biomedical applications. Proliferation, cell shape analysis, focal adhesion, cell-related gene expression, and gene transfection abilities of NIH-3T3 fibroblasts on glass, GO, rGO, MWCNT, double layer of GO and aminated MWCNT, and that of rGO and aminated MWCNT have been systematically evaluated and selected sample results are shown in Fig. 10 [79]. These results show that there are similar proliferation, cell adhesion, spreading patterns, cell and nuclear shapes, and expression levels of adhesion-associated genes (integrin, α -actin, etc.) on these different substrates compared to a control glass substrate. On the other hand, gene transfection efficiency of NIH-3T3 and Hela cells using the transfection reagent Lipofectamine 2000 was up to 2-2.5 times more effective on GO and rGO coated substrates than on control glass.

2.2 Three-dimensional nanoarchitectures from carbon nanotubes for cell growth

3D nanoarchitectures can also be obtained from composites of CNTs with polymers. For example, ice segregation induced self-assembly (ISISA) has been applied to a mixture of CNTs and polymers (Fig. 11) [80]. In the ISISA method, aqueous suspensions of short or long MWCNTs containing different polymers (chitosan, chondroitin sulphate, and gelatin) were subjected to freeze drying, and different architectural and morphological features at the microscale were obtained. 3D materials containing cell-size pores and low surface roughnesses had the highest viability values while those with small pore sizes (relative to cell size) and high surface roughnesses had the lowest viability values. This paper shows that cell viability varies according to the 3D nanoarchitectures of CNT-polymer composites.

3D patterned CNT was synthesized by dropping water onto vertically aligned CNT forest (Fig. 12) [81]. The resulting 3D patterned structure possessed cell-sized cavities and was used directly or after coating with collagen as a platform for cell culture. MSCs were cultured on these materials, and cell viability was tested. The highest cell count at an early stage of cell culture was observed for non-coated, patterned CNT compared to the coated structure and glass. This result can be attributed to the favorable cell adhesion on bare CNT. Hydrophilic CNT forest can also be used for cell cultures [82]. Superhydrophilic forest of CNTs was obtained by exposure of CNT forest to oxygen plasma. In this way, polar functional groups such as CHO, OH, and COOH could be introduced to the CNTs. After introduction of hydrophilic groups on CNTs, the surface of the forest became highly hydrophilic. Human chondrocyte was cultivated on this superhydrophilic forest with the material being found to permit cell growth and adhesion. Expression of a series of mRNA was also tested with superhydrophilic or hydrophobic forest but only a small difference was observed.

2.3 Sensing and controlling cell functions on nanoarchitectures of carbon nanotubes

CNTs have fascinating electronic properties and their high mechanical strengths making them attractive as a scaffold for cell culture with cellular control using an applied electric field. This is especially relevant to neuronal and muscle cells since in living systems they mediate or are controlled by electric fields. CNTs are advantageous for controlling neuronal cells on the basis of their structure and conductive/semi-conductive properties, since neuronal cells form fibrous networks and they are controlled by electronic potentials. Regulation of cell connections, stimulation and sensing of cells, and control of the cell networks are possible by using CNTs [33].

SWCNT can be used as a material for electronically stimulating neurons [83]. Neuronal cells (hippocampal cells from rats) were cultured on a thin film of pure SWCNT [84; 85]. It was shown that spontaneous synaptic activity and cell firing were enhanced. When electrical stimulation was applied via the SWCNT sheet, the signal was delivered directly to cells. The neuronal circuits were stimulated via the SWCNT thin film, which was monitored by patch clamp experiments. When nanocarbons are used in combination with lithographic techniques, further fine tuning of nanoarchitecture and addition of functionality become possible. When CNTs are synthesized on a microscale patterned catalyst, CNT islands of 80 μm dimensions can be fabricated on a device (Fig. 13) [86]. Neuronal cells (cortical cells of rat) could be positioned on the micro-islands of CNT and a higher signal-to-noise ratio for extracellular signals was recorded from the cells on the CNTs compared with the case using titanium nitride electrode. The reasons for observation of enhanced

signal-to-noise ratios are the excellent electrochemical properties of CNTs and improved cell-electrode interactions.

Recently, the effects of a 3D architecture of SWCNTs and electric field on human neuronal cells (neuroblastoma SHSY5Y) has been investigated (Fig. 14) [87]. A 3D architecture of SWCNTs/polymer composite was obtained by grid-assisted deposition. SWCNT/polystyrene beads (PS) 'semicapsules' were prepared by mixing an SWCNT suspension with detergent and PS, and these semicapsules were patterned onto a silicon wafer by grid-assisted deposition. Cell viability and morphological changes in the cells varied according to differences in the 3D architectures.

SWCNTs showed random orientations on the surfaces of the capsules. On the patterned SWCNT capsules, most cells adhered preferentially to the SWCNTs and only a few cells were found on the silicon oxide surrounding the SWCNTs. Cell size and shape could be varied by applying an electric field across the 3D SWCNT structures. An electric field of 1-5 V lead to better adhesion than the control (0 V) and a rather narrow morphology was observed under electric field stimulation. This result indicates that SWCNTs are suitable for neural cell-adhesion and can be used to control cell attachment. Another recent result illustrates the effect of electric field on muscular cell differentiation. For synthesis of cell growth scaffold, dielectrophoresis was used to align CNTs in gelatin methacrylate (GelMA) hydrogels (Fig. 15) [88; 89]. By alignment of CNTs, the conductivity of the gel was increased while its mechanical strength was also slightly increased. A groove-ridge micropattern was created on this gel and C2C12 myoblast was cultured on the fabricated gel. High efficiency of differentiation of the cells on this matrix was observed especially when electronic stimulation was forced at the voltage of 8 V. Differentiation of the cells was confirmed by staining with myosin

heavy chain and F-actin, and by expression of genes related to muscle cell differentiation.

Lateral motion of secretory vesicles of neuroendocrine PC12 cells was restricted on CNT thin film with a nanosized mesh [90]. CNT thin film was synthesized at a water-organic interface. Oxidized SWCNTs having carboxylic acid groups were suspended in water and an organic solvent was added to form a thin film of the oxidized CNT. From observation of this CNT thin film by AFM, it was found that bundles with diameters of 10 nm formed a network of mesh with a size much less than 1 μm . This CNT mesh had a surface roughness comparable to the extracellular matrix which is composed of collagen fibers. The CNT film was coated with PLL to promote cell adhesion, and neuroendocrine PC12 cells were cultured on this coated CNT film. The cultured cells showed similar viability, morphology, growth, proliferation, and differentiation ability compared with those cultured on glass. On the other hand, vesicle motion was restricted in CNT-supported cells compared with control cells on glass. The average coverage area of motion was only 24% of that in the control cells. The average vesicle velocity was reduced to 1/3 in the cells on CNT film compared with that of control cells. Since vesicle motion on cells grown on uniformly evaporated carbon thin films with PLL was similar to that of control cells, it is speculated that the decreased vesicle motion originates from the nano-roughness of 10 nm. Considering that a cell membrane has 5 nm thickness, it must be deformed when placed in the CNT mesh. Although further mechanistic investigation is necessary, deformation of membrane may restrict the activity of membrane proteins that control vesicle movement.

Functional groups on CNTs also have a large effect on the function of neuronal cells. MWCNTs were functionalized either non-covalently through π - π interactions or

covalently through chemical bonds. MWCNTs having ammonium, carboxylic acid, or polyether groups were synthesized. Primary neuronal cells of hippocampal neuronal cultures were prepared from E19 rat embryos and cultured on the CNTs. Different neuronal cell functions such as cell adhesion and network organization were observed from different CNTs. The results may be due to the presence of certain functional groups that can adsorb cell regulating molecules and proteins [91].

When CNTs were internalized into plants and the electron transport ability of CNTs was exploited, a hybrid material with an increased rate of photosynthesis was produced [92]. SWCNTs were coated with DNA, chitosan, or polyvinyl alcohol (PVA) and they were dispersed in water. PVA coated SWCNTs having neutral surfaces could not be internalized in chloroplasts. However, DNA-coated SWCNTs that have an anionic surface and chitosan coated SWCNTs that have cationic surfaces could be internalized to chloroplasts. The internalization of charged wrapped SWCNTs was through a passive uptake mechanism rather than endocytosis, which is similar to the case of mammalian cells. Since SWCNTs have a broad absorption spectrum in the near-infrared region, they do not photo-bleach and have good electron transport capability. The composite materials showed three times higher photosynthetic activity than a control plant, such as spinach or Arabidopsis thaliana leaves. The material could also be used for detection of ROS including NO. Although a similar detection system has been reported for a graphene composite [59], the merit of this material is that it can be internalized to cells, which is not possible with large 2D graphene sheets.

3. Fullerene

Fullerenes are a class of compounds consisting exclusively of sp^2 carbons arranged in a spherical network [93]. Fullerenes can have different dimensions, but the most common derivative consists of 60 carbons, and is known as C_{60} . Various methods for its production have been developed so far, including laser ablation, electric arc discharge, combustion techniques, etc. Of these, the combustion technique is one of the best methods for the production of large quantities. Of the various isomers of fullerene, fullerene- C_{60} is the most widely available and well-studied compound. Although it was discovered earlier than the two other nanocarbons mentioned here (graphene and CNTs), application as a cell culture platform has not been investigated to any significant extent. In addition to its structural significance, fullerene C_{60} possesses the distinction from the other two nanocarbons in that it is a molecule while the other two are nanomaterials. Consequently, fullerene, especially fullerene- C_{60} , is available as a pure compound and its derivatives can be isolated as single well defined compounds while graphene, CNTs, and their derivatives are obtained as structurally poorly defined materials. Several precise-synthetic methods are now available and interesting properties have been found for functionalized fullerenes and their self-assembled structures.

3.1 Nanoarchitectures from fullerenes for cell growth

Although toxicity of nanocarbons has been debated and conflicting results have been reported so far, fullerene- C_{60} has an acceptable biocompatibility. Of the nanocarbons, fullerene has the longest history of research into its toxicity, and the toxicity of water-soluble organo-functionalized fullerenes was first reported in 1993 [94]. It is widely accepted that the size of the aggregates of C_{60} in water suspensions strongly affect its toxicity [28]. Since the previous toxicological results are based

mainly on studies of the toxicity of fullerene derivatives or of composites of fullerenes and detergents it is difficult to evaluate the inherent toxicity of fullerene. However, it has now become possible to prepare and separate fullerene nanoparticles (i.e. aggregates of fullerene molecules) of different size by using centrifugation. As a result the size dependency of the fullerene nanoparticles on cytotoxicity could be evaluated [95]. After centrifugation, the C₆₀ nanoparticles were separated by size in the range from 110 to 180 nm. Of these sizes, inhibition of DNA polymerase and cytotoxicity was greater for the smaller fullerene nanoparticles.

Reports on fullerene for use as a scaffold for cell culture are rare. The one-nanometer sized, non-dimensional structure which produces only fragile assemblies may lead to difficulties in the application of the compound as a scaffold for cell cultures. One solution for production of a 3D scaffold might be vacuum vapor deposition using a mask. Fullerene-C₆₀ was deposited on to microscopic glass coverslips under vacuum through metallic masks with rectangular openings of 100 µm size (Fig. 16) [96]. Several patterned surfaces were formed with differences in height ranging from 130 to 1040 nm. The human osteoblast-like MG63 cells were distributed homogeneously on the surfaces when the height of patterned C₆₀ was less than 330 nm, but localized almost exclusively in the grooves when the height was 1040 nm. Fullerene-C₆₀ mimics the nanoarchitecture of the micropattern which exists in natural tissues. Cell differentiation was observed on the same patterned C₆₀ when human osteoblast-like MG63 cells were cultured.

Another method to make a scaffold composed of C₆₀ is its coating onto a scaffold made from other materials such as polymers. Carbon fiber-reinforced carbon composites (CFRC), materials promising for hard tissue surgery, were coated with a fullerene-C₆₀ layer, and the viability of human osteoblast-like MG63 cells was tested

[97]. On fullerene layers, the cells adhered in numbers from 2.3 to 3.5 times lower than those on control non-coated CFRC or PS dishes. However, their spreading area was larger by 68 to 145% than on the control surfaces. This result suggests that fullerene-C₆₀ may have a moderate biocompatibility compared with that of graphene or CNTs. The electrospinning method is one of the simplest and economical methods for preparing materials as fibers. Nanofibers consisting of poly(L-lactic acid) (PLLA) and C₆₀ were synthesized by this method (Fig. 17) [98]. Water-soluble fullerene nanoparticles were prepared by reaction of fullerene with tetraethylene glycol (TEG) in the presence of LiOH. The fullerene nanoparticles were then mixed with PLLA and the mixture solution was electrospun. The resulting nanofibers of PLLA-fullerene composite was revealed to have a core-shell structure by TEM image and the fibers exhibited fluorescence of fullerene-C₆₀. When the materials were treated with HepG-2 (human liver carcinoma) cells, fluorescence was observed from the cell nuclei. It is thought that fluorescent fullerene nanoparticles were released from the nanofibers and penetrated into the HepG-2 cells, although internalization of fibers was not clearly observed. Nanofibers composed of C₇₀ instead of C₆₀ were also synthesized and larger intensity of fluorescence was observed compared to the case for C₆₀.

3.2 Sensing and control of cell functions using nanoarchitectures of fullerenes

Although few investigations of the use of fullerene as a scaffold of cell culture has been reported so far, biological applications have been well studied [99]. Since fullerene possesses a highly hydrophobic spherical structure, it can bind to therapeutic agents, hydrophobic pockets of proteins or hydrophobic regions of DNA, and so modulate their activity and be used to deliver them into cells. Fullerene can also act as a

radical scavenger so that cells can survive under oxidative conditions. Since fullerene-C₆₀ itself has a highly hydrophobic structure, it is necessary to introduce hydrophilic moieties for work in aqueous phases. In this section, we describe examples of such fullerene derivatives, their self-assembled structures, and biological applications.

Fullerenol, an oxidized fullerene, has many hydroxyl groups substituted on the fullerene molecule making it soluble in water. Among them, only the one with 24 hydroxyl groups is compositionally and structurally well characterized, and has been used extensively for biological experiments. In the case of drug delivery, fullerenol showed distinct differences based on its structure and resulting assemblies compared to CNT. Fullerenol and CNT were conjugated with doxorubicin and cellular uptake was investigated (Fig. 18) [100]. The morphologies of the complexes differed with doxorubicin-fullerenol forming globular 60-80 nm size aggregates and doxorubicin-CNT forming needle-like 300 nm length aggregates. Fullerenol-doxorubicin conjugate was rapidly internalized into the lysosomal compartments of cells while weak internalization was observed for the CNTs into human umbilical vein endothelial cells. This difference might originate from the morphology of the respective aggregates since it has been demonstrated that particles with very high aspect ratios (>20) exhibit minimal uptake into macrophages compared to conventional spherical particles with equal volume. It was suggested that treatment with CNTs resulted in an up-regulation and reorganization (clustering) of $\alpha v \beta 3$ integrin in the endothelial cells which did not occur in the cells treated with fullerenols or those treated without CNTs or fullerenols. The spherical structures of fullerenes and long, thin structures of CNTs lead to quite different biological effects based on their structures and

assembly.

Unique amphiphilic fullerenes, in which the fullerene acts as a hydrophobic moiety, can form specific self-assembled structures. Pentaphenyl fullerene potassium salt ($\text{Ph}_5\text{C}_{60}\text{K}$), in which the fullerene acts as both hydrophobic and hydrophilic parts, can form a self-assembled spherical bilayer vesicle in water (Fig. 19) [101]. The penta-substituted fullerene stabilizes the cyclopentadienide anion leading to the molecules' amphiphilic nature with the hydrophobic hydrocarbon ball linked to a hydrophilic ion. The surface of these sub-100 nm capsular objects can be functionalized by covalent and non-covalent chemical processes. Sub-100 nm capsular 3D nanoarchitectures containing proteins at their surfaces were constructed by covalent and noncovalent multilevel surface functionalization by using the stable and uniform platform of a fullerene bilayer vesicle [102]. The anion of a terminal alkyne tethered penta-aryl fullerene formed the sub-100 nm vesicle in water, and could be functionalized using click chemistry to obtain a biotin-functionalized capsule, followed by noncovalent functionalization with avidin to obtain the protein coated capsule. The biotinylated fullerene vesicle was also applied for anticancer drug delivery in vitro. The sub-100 nm nanoarchitecture allowed delivery of encapsulated drugs into cells.

Fullerene can be adapted for DNA delivery by introduction of amine moieties. Various cationic aminofullerenes exhibited a higher binding ability for DNA than that of simple polyamines (Fig. 20) [103]. Once made water-soluble by the attachment of amine groups, any fullerene compound can bind to DNA and condense it into aggregates at the nano- to micrometer scale. This aggregation ability enhanced cellular uptake of the resulting aggregates. By designing the potential for cleavage of DNA amino groups from the fullerene core or for transformation of the amino groups to

neutral groups, the aminofullerenes **can** release DNA enabling efficient transfection. Tetra-amination of fullerene is one of the simplest and high yielding methods to obtain amphiphilic fullerenes. Since amines can bind to DNA the resulting tetraaminofullerene can be used as a transfection agent. Tetraaminofullerene with a large hydrophobic core of fullerene and side chains bearing a suitable number of ammonium cations stabilizes DNA effectively against nuclease [104]. This stabilization ability enables delivery of DNA in the presence of serum (in which lipid-based transfection agents are less effective) and lead to a 10 times higher efficiency for confluent cells than that obtained using the lipid-based transfection agent, Lipofectin. Gene delivery has also been reported in vivo. Tetraaminofullerene (TPFE) forms globules together with DNA which are sub-micrometer-sized and are therefore an effective size for cellular uptake. Plasma protein effectively stabilized the globules for at least 2 h [105]. TPFE shows higher gene delivery in the liver and spleen than that of the lipid-based transfection agent, Lipofectin, possibly because of π - π interactions between fullerene and nucleobases. The TPFE-DNA complexes were also utilized for therapeutic applications where TPFE was used to effectively deliver insulin plasmid to mice leading to reduced blood glucose levels. **Recently, delivery of siRNA to the lungs of mice was also achieved with TPFE** [106]. Although application of fullerene as a scaffold for cell culture has not been well developed so far, science in the solution phase is well developed compared with the other two **nanocarbons**, graphene and CNTs, and precisely controlled assembly and functions can be obtained.

Conclusion

Research on **nanomaterials** consisting solely of carbon has become a

burgeoning field. It was initiated by the discovery of fullerene-C₆₀ in 1985 and has been enabled by the availability of bulk fullerene production by the combustion technique. There now exist two further nanocarbon materials, CNTs and graphene with respectively 1D and 2D structures. Additionally, porous nanocarbons are available with 3D structures. This nanocarbon can be synthesized by templating using inorganic materials. Therefore, we now have access to carbon nanomaterials of all dimensionalities and they should be investigated for several potential uses including biological applications (as described here).

Living systems are abundant in carbon and this element is the basis of so-called organic chemistry. In humans 18% of our body weight (half our body weight without water) consists of carbon [107]. Considering that the Earth contains only 0.08% by weight of carbon (at the surface of the earth and in the ocean), our bodies can be seen as an extremely carbon-enriched structure, as can most organisms. This is due to the rich and versatile chemistry of the element carbon which can be used to build up complex 3D molecules held together by covalent bonds. The resulting complex 3D shapes allow hierarchical organization through non-covalent interactions eventually leading to the complex supramolecular systems we know as life. Inorganic materials, in contrast, seem to lack such diversity. Thus, the novel carbon materials and nanocarbons can be seen as existing at the boundary between living organic systems and inorganic, inanimate materials. They can possess a high flexibility in structure compared to most traditional inorganic materials and are thus more similar to highly evolved biochemical systems, in part due to their structures being built up from elemental carbon. By using only sp² carbons, nanocarbons with 0, 1, and 2D structures, that is, fullerene, CNTs, and graphene, can be formed. In this review we have shown that further complex

nano-to-macro scale structures can be fabricated by using nanoarchitectonics, chemical functionalization, self-assembly and hybridization of other materials. We have also highlighted that these nano- to macro- spaces are beneficial for cell culture, sensing and control of cellular functions.

Regarding biological systems, they consist of combinations of elements organized into a complex hierarchy. Different tissue types (e.g. connective, epithelial, or neuronal tissue) consist of different cells. These tissues have evolved to be combined within an organism and have characteristic functions. Although cells in a body are initially genetically identical they gain individual forms and functions in the process of cell differentiation. Cell differentiation may also be affected when cells are placed in a space with particular dimensions or properties. If it becomes possible to form such a space then opportunities for advanced tissue engineering should become available. The possibilities and potential benefits of this subject **are** presented in this review.

In this review we have summarized **chemical** functionalization of nanocarbons to form various kinds of 3D nanoarchitectures. Subsequent advances in the science of nanocarbons will be aimed at generating complexity and multiple functionalities by combination and hierarchical assembly of nanocarbons with other materials. The resulting hybrid materials may possess designer properties **for** sensing **or** separation, **and the absorptive** properties of nanocarbons can be combined with particular selectivities [108-111]. For example, combining nanoporous carbon with polymers permitted selective sensing of aniline at the ppm level, which was not possible with either of the starting materials alone [111]. The method of combination and hierarchical assembly of nanocarbons to prepare hybrid composite systems is also applicable **to** materials for cell growth or tissue engineering.

Keywords

Graphene, carbon nanotubes, fullerene, nanoarchitecture, self-assembly, cell

Abbreviations

CFRC, carbon fiber-reinforced carbon composites; CNHs, carbon nanohorns; CNTs, carbon nanotubes; CVD, chemical vapor deposition; FET, field effect transistor; GelMA, gelatin methacrylate; GO, graphene oxide; hESCs, human embryonic stem cells; hMSCs, human MSCs; H₂O₂, hydrogen peroxide; ISISA, ice segregation induced self-assembly; LB, Langmuir-Blodgett; LbL, layer-by-layer; MIMIC, micro-molding-in-capillary; MSCs, mesenchymal stem cells; MWCNTs, multi-walled carbon nanotubes; NIR, near infrared; NO, nitric oxide; PEI, polyethylene imine; PET, polyethylene terephthalate; PDMS, polydimethylsiloxane; Ph₅C₆₀K, pentaphenyl fullerene potassium salt, PLL, poly(L-lysine); PLLA, poly(L-lactic acid); PS, polystyrene beads; PVA, polyvinyl alcohol; RGD, arginine-glycine-aspartic acid; rGO, reduced graphene oxide; ROS, reactive oxygen species; SWCNTs, single-walled carbon nanotubes; TEG, tetraethylene glycol; TPFE, petraaminofullerene; 0D, zero-dimensional; 1D, one-dimensional; 2D, two-dimensional; 3D, three-dimensional (3D).

Acknowledgements

This work was partly supported by World Premier International Research Center Initiative (WPI Initiative), MEXT, Japan and the Core Research for Evolutional Science and Technology (CREST) program of Japan Science and Technology Agency (JST),

Japan.

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Graphical Abstract

Revised.

Research Highlights

- Recent examples of bioactive nanocarbons, graphene, carbon nanotubes, and fullerene, are reviewed.
- Nanoarchitectonics of nanocarbons are shown according to their dimensional classification.
- Methods for control of construction of nano-to-micrometer spaces using nanocarbons are shown.
- Applications of nano-to-micrometer-sized spaces for cell culture are shown.
- Sensing and control of cell activity using nanocarbons are summarized.

Figure Captions

Fig. 1. Nanocarbon-based nanoarchitectonics and their biological applications.

Fig. 2. rGO hydrogel film as cell scaffolds and implantation platforms. a) Preparation of rGO hydrogel film by filtration technique. b) Schematic illustration of cell culturing on the rGO hydrogel film and its application to the implantation platform into rat

subcutaneous dorsum sites.

Fig. 3. Cell differentiation on a fluorinated graphene scaffold. a) Structure of fluorinated graphene. b) Schematic illustration of patterning fluorinated graphene film by printing PDMS. c) Schematic illustration of cell adhesion, morphology change, cell differentiation on the fluorinated graphene film.

Fig. 4. Enhancing stem cell growth and differentiation on graphene and GO scaffold. a) Schematic illustration of multilayer graphene films by LbL assembly. b) Schematic illustration of GO films by LB technique. c) Schematic illustration of protein adsorption on graphene and GO. Insulin denatured on graphene film while GO films showed strong affinity with insulin.

Fig. 5. Graphene-based polyelectrolyte multilayers for neural cell scaffolds. a) Schematic illustrations of LbL preparation of 2D and 3D graphene-polyelectrolyte multilayer. b) Schematic illustrations of neural cell adhesion and neurite outgrowth on graphene-free (left) and graphene-polymer nanofibers (right).

Fig. 6. Acceleration of osteogenic differentiation on a large-scale graphene scaffold formed by chemical vapor deposition. a) and b) Schematic illustration of large-scale graphene scaffold on flat (a) and rough substrates (b), and their cell viabilities. c) Graphene sheet induces osteogenic differentiation of hMSCs.

Fig. 7. Patterned rGO film-based front-gated FET for dopamine detection. a) Schematic

illustration of micropatterning GO films by MIMIC method and reduction of the patterned GO films. b) Schematic illustration of the dopamine detection from PC12 cells on rGO FET.

Fig. 8. Surface nanostructure-dependent cellular growth. a) Preparation of thin film of SWCNT network by phase separation facilitated self-assembly of dispersed carboxylated SWCNT. b) Preparation of rGO film. c) Comparison of cellular growth on the nanocarbon-based scaffolds.

Fig. 9. Controlled growth and alignment of CHO cells on aligned MWCNT sheet. a) Preparation of MWCNT sheet from MWCNT forest produced by solid-state catalytic CVD. b) CHO cells on the aligned MWCNT sheet grow an elongated shape aligned with the MWCNT bundles.

Fig. 10. GO and rGO-based cell scaffolds for adhesion and gene transfection. a) Preparation of GO and rGO substrates. b) Schematic illustration of gene transfection of GFP plasmid into cells on the GO and rGO substrate using Lipofectamine2000.

Fig. 11. MWCNT-based chamber-like 3D scaffold. a) Preparation of 3D MWCNT-based scaffold via ISISA process with different polymer suspensions (chitosan, chondroitin sulfate or gelatin). b) Illustration of chamber-like 3D scaffold. c) Illustration of cell culture on the 3D scaffold.

Fig. 12. Cell-size pore cavity patterning of vertically aligned MWCNT arrays.

Fig. 13. CNT-based multi-electrode array for recording device of extracellular signals.

a) Schematic illustration of multi-electrode array and micrometer-sized CNT-based electrode. b) Cell culture and extracellular signal recoding on the multi-electrode array.

Fig. 14. Grid-assisted patterning of SWCNT/PS scaffold. a) Schematic illustration of grid-assisted deposition of the SWCNT suspension. b) Image of patterned SWCNT/PS grid. c) Cell adhesion on the patterned SWCNT/PS grid. d) Electric field induced morphological change of the cell.

Fig. 15. Myotube differentiation induced by electrode-attached aligned MWCNT-hydrogel hybrid. Schematic illustration of dielectrophoresis-based alignment of MWCNTs and electrical stimuli-induced myotube differentiation. IDA, interdigitated array.

Fig. 16. Groove-selective cell adhesion and growth on micropatterned fullerene layers. a) Schematic illustration of micropatterned fullerene layers prepared by C₆₀ evaporation technique. b) Cells grow selectively on **micropatterned** grooves. Height of C₆₀ layers are ca. 1 μm .

Fig. 17. PLLA nanofiber encapsulated with water-soluble fullerene nanoparticles by blend electrospinning. a) Preparation of water-soluble fluorescent fullerene nanoparticles. b) Schematic illustration of formation of PLLA nanofiber encapsulated with fullerene nanoparticles by electrospinning method. c) Cell culture on the

fullerene/PLLA nanofiber scaffold. Intense red fluorescence derived from fullerene nanoparticles was observed in the nuclei, whereas no fluorescence was observed on the PLLA fiber scaffold lacking fullerene nanoparticles.

Fig. 18. Cellular uptakes of carbon cluster-based doxorubicin carriers. a) Preparation of fullerenol-doxorubicin particles. b) Preparation of SWCNT/pyrene-doxorubicin aggregates. c) Schematic illustration of cellular uptakes of the carbon cluster-based doxorubicin carriers.

Fig. 19. Formation of bilayer vesicles made of penta-substituted fullerene anions. a) Structure of penta-substituted fullerene anions. b) Schematic illustration of fullerene bilayer vesicle.

Fig. 20. Delivery of DNA via tetracationic aminofullerene derivatives. a) Structures of tetracationic aminofullerene derivatives. b) Schematic illustration of submicrometer-sized DNA-fullerene particles.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

**Acid-treated
functionalized MWCNT**

Chamber-like 3D scaffold

**Pore size & surface roughness-
dependent cell viability**

Figure 12

Figure 13

a

b

Figure 14

Figure 15

a

Figure 16

Figure 17

a

b

c C₆₀ fluorescence from nuclei

Figure 18

Figure 19

R = H,
 $-(\text{CH}_2)_n\text{CH}_3$ ($n = 0-19$),
 $-(\text{CH}_2)_6\text{C}\equiv\text{CH}$
 $-(\text{CF}_2)_8\text{CF}_3$

b

Figure 20

Biographical Sketch

Dr. Waka Nakanishi received her Ph.D. in chemistry (2005) from the University of Tokyo. She became an assistant professor at Chiba University in 2005, became a Research Fellow in Bilateral Programs of Japan Society for the Promotion of Science (JSPS) to visit University of Alberta in 2007, and then became a lecturer in Tohoku University in 2008. From 2013, she joined the National Institute for Materials Science (NIMS). Her research **areas are** synthetic chemistry, physical organic chemistry, and nano-bioscience. Her current interests include synthesis of conjugated molecules and control of their structures for application to material sciences and biology.

Dr. Kosuke Minami received his B.Sc. in biology (2008) and Ph.D. in chemistry (2013) from the University of Tokyo. He started working as a postdoctoral fellow in National Institute for Materials Science (NIMS) from 2013 and became

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Dr. Lok Kumar Shrestha received his Ph.D. from Yokohama National University, Japan, in 2008. He is currently a MANA Scientist at the World Premier International (WPI) Research Center for Materials Nanoarchitectonics (MANA) of the National Institute for Materials Science (NIMS). His current research interests include production of self-assembled fullerene crystals from zero to higher dimensions and designing nanospaces within these crystals for energy storage and sensing applications.

Dr. Qingmin Ji received her Ph.D. (2005) in chemistry from the University of Tsukuba. She started working as a postdoctoral fellow in National Institute for Materials Science (NIMS) from 2006 and became MANA scientist at World Premier International (WPI) Research Center for Materials Nanoarchitectonics (MANA) in 2011. Her research currently focuses on the formation of layer-by-layer films and the application of mesoporous structures for delivery systems.

Dr. Jonathan P. Hill received his Ph.D. degree from Brunel University, U.K. in 1995. He is currently subgroup leader of the Supramolecules Group at the National Institute for Materials Science. Current research interests include synthesis and properties of tetrapyrroles and their supramolecular manifolds as well as unusual methods for preparing organic nanomaterials.

Prof. Katsuhiko Ariga received Ph.D. degree from the Tokyo Institute of Technology, Japan. He is currently the Director of Supermolecules Group and Principal Investigator of World Premier International (WPI) Research Center for Materials Nanoarchitectonics (MANA) at the National Institute for Materials Science (NIMS). His research field is based on **supramolecular** chemistry and surface science, including the boundary research areas of organic chemistry, physical chemistry, biochemistry, and materials chemistry. His major interests are the fabrication of novel functional nanostructures based on molecular recognition and self-assembly including Langmuir-Blodgett films, layer-by-layer films, and mesoporous materials.