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In Vivo Remote Control of Reactions in *Caenorhabditis elegans* by Using Supramolecular Nanohybrids of Carbon Nanotubes and Liposomes

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Abstract: *A supramolecular nanohybrid based on carbon nanotubes and liposomes that is highly biocompatible and capable of permeation through cells is described. The nanohybrid can be loaded with a variety of functional molecules and is structurally controlled by near-infrared laser irradiation for the release of molecules from the nanohybrids in a targeted manner via microscopy. We implemented the controlled release of molecules from the nanohybrids and demonstrated remote regulation of the photoinduced nanohybrid functions. As a proof of principle, nanohybrids loaded with amiloride were successfully used in the spatiotemporally targeted blocking of amiloride-sensitive mechanosensory neurons in living Caenorhabditis elegans. Our prototype could inspire new designs for biomimetic parasitism and symbiosis, and biologically active nanorobots for the higher-level manipulation of organisms.*

Nanorobotics is an emerging technological field that involves creating machines or robots, the elements of which are based on chemical or biological molecular units at the nanometer scale.[1] Self-assembly techniques, in which a variety of molecular components are combined into a customized shape through interaction with functional molecules, have proven to be extremely versatile in the development of high-performance molecular structures.[1,2] Many research groups have attempted the construction of self-organized nanorobots, such as DNA nanoworkers,[3] metal-based micro/nanobots,[4,5] and biomolecular motor-powered nanohybrids.[6] Recently, we demonstrated that a new type of supramolecular system comprised of carbon nanotubes (CNTs) and liposomes enabled the directed transport and controlled release of carrier molecules, and supported enzymatic reactions in a desired location.[7] However, the applications of our earlier system of electrical train-like nanorobots, based on a CNT and temperature-responsive liposome, were limited to microfluidic devices. There is currently great interest in understanding and achieving remote operation of functional nanorobots in physiological environments.[8,9] Furthermore, analysis of the biocompatibility and multifunctionalization of nanocarbon materials with bioactive molecules will be a milestone for integrating these new materials with numerous biological applications.[10,11]

Organisms have developed surprising abilities through long evolutionary processes. Examples include parasitism and symbiosis, which are sophisticated relationships between different organisms in which the parasite or symbiont coexists with their host.[12–14] Some parasites, such as *Leucochloridium paradoxum*[12] and *Paragordius tricuspidatus*,[13] are known to control the dynamic behavior of the host organisms by manipulating motor nerves via intercellular chemical signaling. In some cases, symbionts provide their hosts with significant advantages, such as trophallaxis and protection through biochemical communication.[14] If parasitism and symbiosis could be artificially established, it would lead not only to the development of the ultimate synthetic biological system, but also to a deeper understanding of biological activities and the phenomena of life.

In the last decade, there has been great progress in technologies for the remote control of organisms through physical manipulation.[15–17] In particular, methods involving lasers have proven useful for the control of biological functions in living organisms through simple manipulation with laser beams (which provide advantages such as high directivity, ease of focus on the targets, and fast delivery).[18–21] Ultraviolet (UV),[18] short-wavelength visible (Vis),[19] and infrared (IR)[20] light are generally used in optical techniques for the control of biological functions in organisms. However, these types of light do not efficiently penetrate biological tissues. It is well known that near-infrared (NIR) rays can penetrate tissues because biological systems are relatively transparent to these wavelengths of light (650–1100 nm).[21] We previously developed NIR-laser-driven nanomodulators for the regulation of gene expression[21] and remote control of the electrical current in living cells.[22] Physicochemical activation/inhibition of organic processes is the next key step for the manipulation of biological systems through the construction tools inspired by parasitism and symbiosis.

In this study, we demonstrated the following: 1) supramolecular nanohybrids based on CNTs and liposomes show low cytotoxicity; 2) synthesized nanohybrids can easily penetrate cells; and 3) the nanohybrids can absorb and convert NIR light into thermal energy to facilitate the controlled-release of substrates. The application of these combined

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results demonstrate that spatiotemporal control of the obstruction of amiloride-sensitive mechanosensory neurons in the model host *Caenorhabditis elegans* (*C. elegans*) can be successfully achieved by exploiting the physicochemical characteristics of photo-induced nanohybrids. Therefore, these nanohybrids have significant potential for the advanced photochemical manipulation of biological behaviors in vivo and the development of progressive drug-delivery systems. Our novel design for light-driven nanohybrids will enable the remote and non-invasive inhibition and/or stimulation of a wide range of biological targets in deep tissue owing to the transparency of biological tissues to NIR light and the directed distribution of functional molecules into the cells/tissue. This study is also an important first step towards the creation of biomimetic parasitism and symbiosis by using biologically active nanorobots as parasites and/or an endosymbionts on the nanoscale.

To construct an effective bioinspired parasitic and symbiotic system, CNT- and liposome-based supramolecular nanobiomachines were synthesized by using avidin–biotin interactions and self-assembly techniques (Figure 1 a).[7] Avidin-PEG_{2000}-PL-functionalized SWNT complexes (avidin-PEG_{2000}-PL-SWNT) could be spontaneously combined with biotin-PEG_{2000}-PL and NBD-PE molecules to form a functionalized liposome (PEG_{2000} = polyethylene glycol of molecular weight 2000, PL = phospholipid = N-linked 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, SWNT = single-walled CNT, PE = N-linked 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, NBD = 7-nitro-2-1,3-benzoxadiazol-4-yl). By using fluorescence microscopy and transmission electron microscopy, we optimized the mixing ratios between the SWNT and liposome, and confirmed the generation of supramolecular nanostructures (Table S1 and Figures S1, S2 in the Supporting Information).

The biocompatibility of our CNT-liposome nanohybrids is one of the most important aspects, not only for the control of reactions in living organisms, but also for future clinical and biomedical applications. We thus tested the viability of human HeLa cancer cells that had been pre-incubated with the nanohybrids at the following concentrations (SWNT = 0, 10, and 25 $\mu g\,mL^{-1}$, lipid in liposome = 0, 60, and 150 μM, respectively) by using WST-1 assays (Figure 1 b). Likely as a result of the water-dispersing properties and the high biocompatibility of PEG[23] at the interfaces of the SWNTs and liposomes, over 77% of the cells remained viable following treatment for all concentrations tested. In addition, we also investigated the internalization and distribution of nanohybrids in the HeLa cells by using fluorescent NBD-labeled nanocomplexes (SWNT = 10 $\mu g\,mL^{-1}$, lipid in liposome = 60 μM; Figure 1 c). HeLa cells were seeded in 35 mm glass-bottom dishes and incubated with the nanohybrids for 24 h. Intracellular uptake of the NBD-labeled nanohybrid was assessed by using laser confocal microscopy. Fluorescence emission from both intra- and extracellular NBD-labeled nanohybrids was observed. We confirmed that the nanohybrids were mainly localized inside cells by three-dimensional analysis (Figure S3). Notably, the NBD-labeled nanohybrids demonstrated a higher degree of cell permeability than the NBD-labeled liposomes, since higher fluorescence

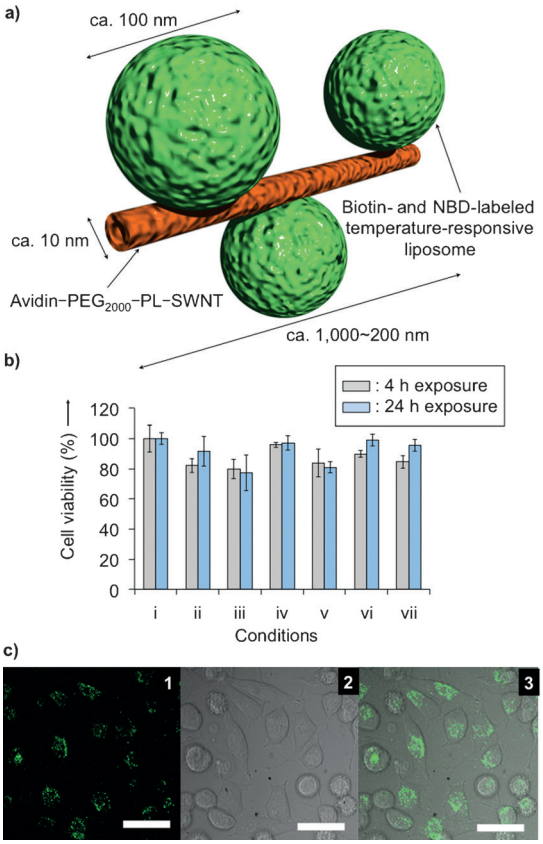

Figure 1. CNT-liposome supramolecular nanohybrids. a) Schematic illustration of the nanohybrids. b) WST-1 assays in HeLa cells after 4 and 24 h exposure to the nanohybrids. Conditions: i) SWNT = 0 $\mu g\,mL^{-1}$, lipid in liposome = 0 μM, ii) SWNT = 10 $\mu g\,mL^{-1}$, lipid in liposome = 60 μM, iii) SWNT = 25 $\mu g\,mL^{-1}$, lipid in liposome = 150 μM, iv) SWNT = 10 $\mu g\,mL^{-1}$, lipid in liposome = 0 μM, v) SWNT = 25 $\mu g\,mL^{-1}$, lipid in liposome = 0 μM, vi) SWNT = 0 $\mu g\,mL^{-1}$, lipid in liposome = 60 μM, and vii) SWNT = 0 $\mu g\,mL^{-1}$, lipid in liposome = 150 μM. c) Confocal microscopy images (λ_{ex} = 488 nm, λ_{em} = 510 nm) of HeLa cells after incubation with NBD-labeled fluorescent nanohybrid for 24 h (SWNT = 10 $\mu g\,mL^{-1}$, lipid in liposome = 60 μM). Fluorescence (1), Differential interference contrast (DIC; 2), and combination of fluorescence and DIC images (3) are shown. Scale bars, 50 μm. Magnification = 60×.

intensity was seen in cells incubated with the nanohybrids (Figure S4). These results strongly suggest that the nanohybrids show effective and safe cellular interactions.

The supramolecular CNT-liposome nanohybrids expressed a powerful photothermal conversion response to laser triggering (Figure S5). The thermal behavior of rhodamine B (RhB) in a solution of nanohybrids was observed under NIR irradiation via real-time fluorescent microscopy (Figure S5 a). Ultrafast quenching of the fluorescence of RhB was observed (within 0.03 s) upon irradiation with a NIR laser (808 nm; see movie M1 in the Supporting Information). The quenching of RhB occurred distinctly in the center of the image owing to irradiation with the highly focused laser beam (diameter of laser spot = 50 μm) from the objective lens (×20). Rapid thermal convection was also monitored during laser irradiation. The fluorescence of RhB was restored to the

original intensity immediately (within 0.03 s) after discontinuation of the irradiation. For a solution without nanohybrids, we confirmed that the RhB fluorescence was not quenched in this laser power range, thus indicating that there was no change in the temperature (Figure S5 b). The temperatures resulting from laser powers of 301, 413, and 564 mW (power densities were 153, 210, and 287 $\mu W\,\mu m^{-2}$, respectively) increased from the 25 °C to 31 °C, 43 °C, and 51 °C, respectively (Figure S5 c).

Controlled release of substrates from supramolecular nanohybrids and enzymatic reactions were easily manipulated through external laser-beam irradiation (Figure S6). Previously, we demonstrated that the nanostructures of liposomes can be effectively destroyed at temperatures greater than 42 °C by adjusting the chemical composition and manipulating the powerful photothermal conversion effect (heat energy) of the CNT.[7] For the initial system designed to achieve control of reactions in living organisms, we employed the reaction of β-galactosidase (β-Gal) from *E. coli* as a model reaction. Since the destruction of liposome proceeds by photothermal energy conversion, the fluorescein di-β-D-galactopyranoside (FDG) is released from the SWNT, reacts with β-Gal to form fluorescein, and the resulting increase in fluorescent emission can be monitored. We observed an overall bright-green fluorescence derived from the β-Gal enzymatic reaction as soon as NIR laser irradiation was initialized (Figure S6 a and movie M2 in the Supporting Information). In control experiments without nanohybrids, we did not observe any fluorescence emission with laser powers ranging from 301–564 mW (Figure S6 b and S6 c). The fluorescence profiles are shown in Figure S6d at three different laser powers [301, 413, and 564 mW (power densities were 153, 210, and 287 $\mu W\,\mu m^{-2}$, respectively). In addition, the fluorescence intensity increased in a laser-power-dependent manner. These results clearly demonstrate that β-Gal enzymatic activity can be remotely controlled with laser-triggered nanohybrids.

In the current investigation, we used *C. elegans* as a model host organism to construct a bioinspired parasitic and symbiotic system because this organism is often used for studying molecular biology. Indeed, the regulatory mechanisms of both gene function and neuronal activity in *C. elegans* are well known.[24] Amiloride is one of the best-known blocking agents for degenerin (DEG) and epithelial sodium channels (ENaCs), which have diverse physiological functions.[25] Consequently, amiloride strongly affects the mechanosensory neurons that express the amiloride-sensitive DEG/ENaC channel proteins in *C. elegans*.[26] Representative mechanosensory neurons [posterior lateral microtubules left (PLML) and right (PLMR)] are located in the tail of *C. elegans*. When PLMs receive physical stimulation, the worms can be induced to move forward via command interneurons. Therefore, we encapsulated amiloride into the nanohybrids in order to monitor the dynamic behavior resulting from a simple model biological reaction through inhibition of the mechanosensory neurons in *C. elegans* (Figure 2 a). The nanohybrids were injected into the body cavity of the *C. elegans* by microinjection, and an adequate distribution of the NBD-labeled fluorescent nanohybrids throughout the body was subsequently confirmed (Figure S7). The loading of amiloride molecules onto the nanohybrids was calculated to be about 11 $\mu g\,mL^{-1}$ (Figure S8). After laser irradiation of the tail, the worms immediately moved forward owing to the photon pressure derived from the focused laser beam[19] [564 mW (287 $\mu W\,\mu m^{-2}$)]. It was noted that irradiated worms became gradually sluggish from repeated laser irradiation to their tails (Figure 2 b and movie M3 see in the Supporting Information).

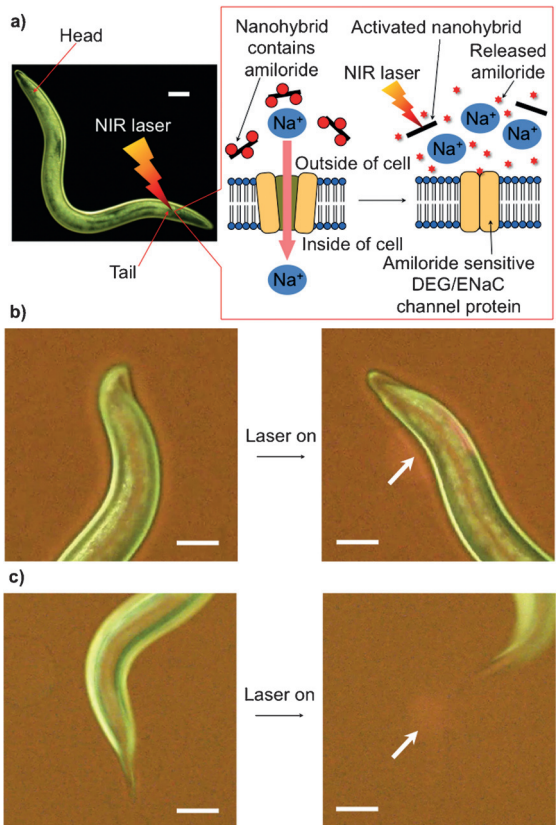

Figure 2. Blocking of amiloride-sensitive sodium cannels in living *C. elegans* by using laser-induced nanohybrids. a) Schematic illustration of the remote control of biological activities in *C. elegans*. b) Dynamic behavior of *C. elegans* with laser-induced nanohybrids encapsulating amiloride (SWNT = 100 $\mu g\,mL^{-1}$, lipid in liposome = 601 μM, amiloride = 11 $\mu g\,mL^{-1}$). c) Control experiment without nanohybrids (M9 buffer). White arrows represent the location of laser irradiation. Scale bars, 50 μm.

Ultimately, the worms completely stopped responding to the laser, likely due to the release of excess amiloride molecules. In the comparable control experiments (nanohybrids without amiloride, liposomes encapsulating amiloride, and M9 buffer without nanohybrids), the worms vigorously moved forward after laser irradiation [> 186 mW (95 $\mu W\,\mu m^{-2}$)] and they continued to quickly move forward despite the repetition of laser irradiation (Figure 2 c and S9). Therefore, we believe that the laser-induced nanohybrids encapsulating amiloride mediated the obstruction of amiloride-sensitive mechanosensory channels in the PLMs of *C. elegans*. Furthermore, controls with CNT-liposome nanohybrid without amiloride and NBD did not alter worm movement within 5 days after an

injection (Figure S10). We also observed that all of the worms microinjected laid healthy eggs. Liposome without amiloride also did not affect the viability and fertility of *C. elegans* at all (Figure S10). Meanwhile, NH_2-PEG_{2000}-PL-SWNT showed relatively low biocompatibility in *C. elegans* although the fertility of the worms was very high (Figure S10). These results clearly indicate that nanohybrids function efficiently in the host organisms as a powerful laser-driven "nanoparasite."

In the present study, we developed light-driven nanohybrids composed of CNTs and liposomes through simple self-assembly technology for the in vivo remote control of bioreactions in *C. elegans*. At the molecular level, our nanohybrids could encapsulate functional molecules, such as fluorescent dyes and protein-channel inhibitors, inside heat-sensitive liposomes. In addition, the nanohybrids showed relatively low cytotoxicity in in vitro biocompatibility assays. Furthermore, the nanohybrids facilitated the generation of heat for the controlled-release of substrates from their nanoconstructs when irradiated with a NIR laser. As demonstrated here, the laser-induced nanohybrid enabled the remote control of mechanosensory neurons in *C. elegans*. This work illustrates the first approach to the spatiotemporal remote control of the biological reactions of *C. elegans* by using an artificial nanobiomachine, which exhibits the attractive features of the physicochemical properties of CNTs and liposomes. The application of this technology to a broad range of sensory systems, such as visual organs,[27] the auditory apparatus,[28] and thermal sensations,[22,29] is easily foreseeable. In addition, the use of biological tissue-permeable NIR lasers would be beneficial for remote and non-invasive inhibition and/or stimulation of a wide range of biological targets.

Keywords: carbon nanotubes · controlled delivery · liposomes · nanotechnology · neurons

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