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研究課題名（和文）Externally Triggered Nanomachine for Breast Cancer Theranostics

研究課題名（英文）Externally Triggered Nanomachine for Breast Cancer Theranostics

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研究成果の概要（和文）：外部活性化用に設計されたプログラマブルなナノデバイスは、がんの診断と治療を促進するために開発されました。炭素ナノドットを用いた人工ナノ酵素は、テラノスティック・ナノマシンの多目的プラットフォームとして注目されました。このプラットフォームは、NIR照射により乳がん細胞の増殖を抑制し、その転移移行を調節する効果を示し、フォトサーマルおよびフォトダイナミック療法の応用が示唆されています。酸素供給量の正常な条件下では、ナノ酵素は転移移行を調節し、さまざまな細胞アッセイで生体適合性を示します。開発されたナノデバイスは、腫瘍微小環境を再構築する能力を持ち、固形悪性腫瘍の効果的な受容性療法の機会を提供します

研究成果の学術的意義や社会的意義
The scientific significance is underscored by the advancement of innovative programmable nanodevices which serve as a versatile theranostic platform, offering effective strategies for cancer treatment. The social significance is tied to the achievement of enhanced cancer diagnosis and medication.

研究成果の概要（英文）：Programmable nanodevices engineered for external activation were designed to facilitate cancer diagnosis and therapeutic treatment. Artificial nanozyme employing carbon nanodots was highlighted as a versatile platform for theranostic nanomachines. It integrates logic control systems, leveraging fluorescent responses to chemical or biological stimuli. This platform exhibits effectiveness in inhibiting breast cancer cells and regulating their metastatic migration through NIR irradiation, suggesting applications in photothermal and photodynamic therapy. Under normoxic conditions, the nanozymes regulate metastatic migration while demonstrating biocompatibility across various cellular assays. The therapeutic dosage was remarkably low, with no discernible adverse effects on healthy cellular pathways. The developed nanodevices were capable to reshape the tumor microenvironment, thereby establishing a therapeutic opportunity for effective adoptive therapy in treating of solid malignancies.

研究分野：Nanotechnology

キーワード：Nanomedicine　Nanomachines　Drug delivery systems　CAR T Immunotherapy　Adoptive therapy　Non invasive chemotherapy　Nanozyme　Cancer

様　式　Ｃ－１９、Ｆ－１９－１（共通）

１．研究開始当初の背景

The exploration of nanomachines for cancer theranostics represents a promising frontier within the realms of nanotechnology and nanomedicine. These minuscule devices, engineered at the molecular and nanoparticle scales, hold the potential to convert various forms of energy—such as radiation or chemical stimuli—into mechanical forces. Consequently, they possess the capability to detect, diagnose, and treat cancer with unparalleled precision. Among these nanomachines, light-powered nanoconverters stand out as a particularly intriguing category. Comprising light-sensitive materials, such as specific photoresponsive nanoparticles or assemblies of photosensitizers, these nanoconverters offer enticing prospects for advancing biomedical nanotechnologies. Nevertheless, further investigation is imperative to refine their performance and fully exploit their capabilities. For instance, a notable contribution by Loukanov et al. in 2018 (10.1021/acs.jpcc.7b11779) introduced a nanoconverter crafted from non-toxic nanoparticles—specifically ultrasmall carbon nanodots and iron oxide. This nanoconverter was engineered to harness near-infrared light, transforming it into highly reactive oxygen species within tissues. This conversion mechanism induces apoptosis in cancer cells, rendering it a potent therapeutic tool. Initial experiments revealed minimal cytotoxicity towards healthy cells, underscoring its safety profile. Upon accumulation within the tumor microenvironment, these nanoconverters can infiltrate malignant cells via endocytosis, triggering apoptosis through chromosome destruction. Furthermore, there exists potential for nanoconverters to serve as a versatile platform, capable of integration with biomolecules such as DNA/RNA, antibodies, or biomarkers. This synergistic approach could yield biomimetic nanomachines endowed with the ability to recognize and selectively target cancer cells through biorecognition mechanisms. However, the intricate design of photoresponsive nanomachines necessitates engineering at the single-molecule level. Moving forward, research efforts should focus on amalgamating the distinctive attributes of nanoconverters with light-driven biomolecules. This endeavor aims to fabricate light-powered nanomachines with heightened cancer selectivity and cytotoxicity, while simultaneously minimizing adverse effects on healthy tissues.

２．研究の目的

The primary objective of this research was to engineer externally triggered anticancer light-powered nanomachines tailored for potential application in breast cancer theranostics. These nanomachines are intricately designed using specific nanomaterials as energy converters for photodynamic therapy, coupled with biomolecules such as DNA, RNA, antibodies, biomarkers, or biomimetic molecules for precise cancer recognition. These assemblies are interconnected through photo-switch moieties, enabling the induction of cytotoxicity in cancer cells upon irradiation with near-infrared light within the "water window." This wavelength range ensures minimal absorption and scattering in biological tissues, allowing for deep tissue penetration and targeted therapy. Importantly, the nanomachine's design also incorporates mechanisms for self-destruction if inadvertently taken up by normal healthy cells, thereby minimizing the possible harmful side-effects.

３．研究の方法

3.1. *Engineering, synthesis, purification and analysis of biocompatible photoresponsive nanomaterials*

Bottom-up approaches are employed for synthesizing nanomaterials, wherein nanoparticles are constructed from smaller components. Natural compounds such as amino acids, sugars, amines, and vitamins often serve as precursors, with green chemical synthetic methods utilized to minimize toxic by-products. Biocompatible nanomaterials encompass iron oxide nanoparticles, gold nanoparticles, silica, and various carbon-based nanomaterials, including ultra-small carbon nanodots, polymeric, lipid, and hybrid nanoparticles. Hybrid nanoparticles consist of combinations of gold, silica, and carbon entities functionalized with organic ligands. Polymers employed must be biodegradable, exemplified by PLGA (poly-lactic-co-glycolic acid). Photoresponsive components may include light-responsive aromatic domains or the polymer moiety of the nanoparticles, as well as the localized surface plasmon resonance of the gold nanoparticle shell. The quantum yield (QY) was acquired by the absolute PL QY measurement system. Monoclonal antibodies, such as anti-human CD4 and anti-human CD8, etc., peptides (e.g., avidin, streptavidin, etc.), and small molecules, are chosen for their ability to selectively bind to biomarkers or overexpressed receptors on cancer cells. Additionally, single-stranded thiol-terminated DNA sequences, known as aptamers, are

engineered with specific dendritic-dimensional structures. These aptamers are further modified with spacers and amino-groups at thymidine pyrimidine bases to enhance resistance to nucleases. To facilitate drug delivery, messenger RNA is synthesized using the TakaraIVTpro mRNA Synthesis system and incorporated into nanosystems. Plasmids containing the ZsGreen gene are amplified via PCR amplification for experimental purposes. Polymer conjugation to nucleic acids (mRNA) often relies on electrostatic interactions between positively charged biodegradable polymer molecules and negatively charged mRNA oligonucleotides. Hydrophobic aromatic domains within carbon nanodots are typically attached to their hydrophilic oxidized shell via covalent bonds. Thiol-terminated DNA aptamers are conjugated to the surface of gold nanocarriers through thiol-conjugation chemistry. Formation of protective lipid layers in drug-delivery nanoparticles is achieved through non-covalent hydrophobic interactions. Avidin or streptavidin are linked to biodegradable polymers via carbodiimide chemistry (EDC/NHS). Monoclonal antibodies (CD4, CD8, anti-HER2, anti-EGFR etc.) are attached to nanomachines through biotin-streptavidin (avidin) interactions rather than covalent bonds. Nanomaterials were purified from the reaction mixture primarily through precipitation, centrifugation, dialysis, filtration, or size exclusion chromatography. Analysis of nanoparticle size distribution and morphology was conducted using dynamic light scattering (DLS), transmission electron microscopy (TEM), scanning electron microscopy (SEM) equipped with energy-dispersive X-ray (EDX) detector, and atomic force microscope (AFM) with commercial SPM controller. Zeta potential measurements provided data on the surface charge of the nanoparticles. Optical, catalytic, and electrical properties, as well as surface plasmon resonance, were assessed through absorbance, fluorescence, and phosphorescence spectroscopy. Raman spectra were measured with a Laser Raman WiTec Alpha 300 spectrometer using the green exciting radiation (532 nm) from an Ar^+ ion laser. Purity was confirmed via spectrophotometry and high-performance liquid chromatography (HPLC). Additionally, infrared spectroscopy, fluorescence resonance energy transfer (FRET), and nuclear magnetic resonance (NMR) techniques were employed to study the functional groups and structure of organic materials.

3.2. *Investigating of the therapeutic efficacy of nanomachines on the cancer cells*

The cytotoxicity of externally triggered theranostic nanomachines on cancer cells (HCC1954, B16-F1, B16-F10, etc.) was evaluated using the terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay. Cells were cultured in appropriate mediums supplemented with 5% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2 (CO_2-incubator). Flow cytometry was employed for multi-parametric analysis of the cultured cells. The TUNEL assay utilized an in-situ cell death detection kit. Concentration-dependent toxicity of the nanomaterials on normal human cells (HepG2 hepatocytes) was assessed. The cytotoxic inhibition of cancer cells by the nanomachines was studied using the MTT cell viability assay. Mitochondrial and sub-mitochondrial particles (SMP) ATPase activity was determined by measuring inorganic phosphate (Pi) released from ATP. The biocompatibility of illuminated nanomachines was confirmed by assessing ATPase activity of intact mitochondria and sub-mitochondrial particles, diamine oxidase (DAO) activity in liver and kidney fractions in dark mode, MTT assay, and on non-illuminated adenocarcinomic human alveolar basal epithelial cells (A549) and human liver cancer cells (HepG2). Cancer cell inhibition was studied using an inverted confocal microscope. Time-lapse imaging experiments captured multistage points using an automatic stage controlled by NIS-Elements software. The scratch assay was utilized to study the inhibition of cancer cell migration. Female Wistar rats aged 4–6 weeks, with an average body weight (BW) of 100 ± 20 g, were used for in vivo experiments. The animals were kept under standard conditions with a room temperature of 22 ± 2°C, 50–60% humidity, and a 12:12 light–dark cycle. Protein content was determined using the Lowry method with bovine serum albumin as a standard. Statistical analysis was performed with data expressed as mean ± standard error of the mean (SEM) of at least four independent experiments in three parallel samples. Differences between nanoparticles-treated samples and untreated controls were tested by one-way analysis of variance (ANOVA) followed by Tukey test with treatment as a factor.

4．研究成果

Programmable nanodevices, designed for external activation, have been developed as agents for cancer theranostics. These include engineered nanozymes, light-triggered Janus nanomotors, and nanosnowflakes, serving as versatile platforms for nanomachine construction. They have demonstrated efficacy in inhibiting breast cancer cells and regulating their metastatic migration through near-infrared (NIR) irradiation. The nanozyme shows promise for applications in photothermal and photodynamic therapy.

Furthermore, these theranostic nanomachines have shown the ability to deliver cargo such as drugs and mRNA, making them suitable for chimeric antigen receptor T cell-based immunotherapy. Additionally, the nanodevices effectively obstruct suppressor cells within the tumor microenvironment, creating a potential therapeutic opportunity for effective treatment. Below, we discuss the details of these theranostics nanomachines.

► *Theranostics nanozymes*. The efficacy of histidine-containing carbon nanodots as nanozymes activated by visible light was demonstrated. The use of visible electromagnetic irradiation (in the range of 400-500 nm) was particularly advantageous due to its safety compared to ultraviolet light. The successful incorporation of pyrrolic nitrogen doping (from the imidazole group) into the aromatic domains enabled the nanozyme to achieve enhanced photosensitization reactions, leading to increased singlet oxygen production. This strategy was based on the recognition that pyrrolic nitrogen serves as the primary site in the nanozyme for oxygen adsorption, facilitating a crucial reduction in the distance between the C-dots surface and oxygen molecules. The higher content of pyrrolic nitrogen in the designed strategy shortened the distance between the fluorescent nanoparticle and dissolved oxygen molecules, a key factor in improving catalytic photosensitization reactions and enhancing the cytotoxicity of the nanozyme within cancer cells under light irradiation. Increased nitrogen doping into the nanozyme lattice affected the energy levels and activation of its singlet and triplet states, directly influencing the oxygen photosensitization reaction. Due to the heterogeneity in structure and size of the nanoparticles, precise determination of the exciting energy states experimentally was challenging, akin to molecules. Notably, the nanozyme exhibited both standard and upconversional phosphorescence (as shown on Figure 1).

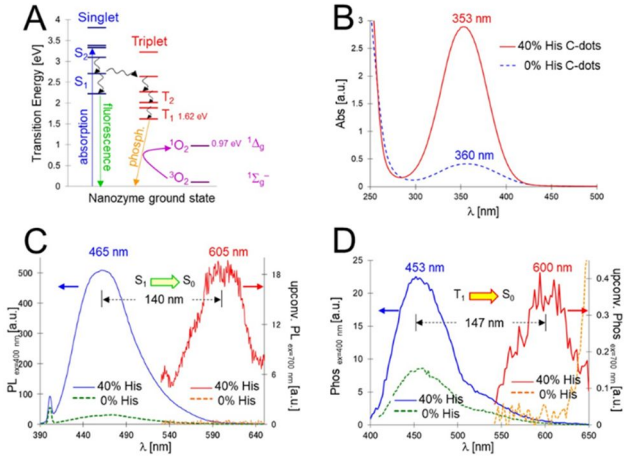

Figure 1. Optical properties of the nanozyme. (A) Jablonski diagram indication of the electronic state and their relative energy levels and the production of singlet oxygen. (B) The bathochromic absorption effect of the nanozyme (solid red line) and its histidine-free counterpart as control experiment (dashed blue line). (C) Down-conversion (solid blue line) and upconversion (solid red line) fluorescence of the nanozyme. (D) Same effect is demonstrated with the down-conversion and upconversion phosphorescence, where the maximum peaks of the nanozyme are located at 453 nm (blue solid line) and around 600 nm (red solid line).

The engineered artificial nanozymes provide a versatile platform for the development of theranostic-programmed nanomachines. Confocal microscopy images in Fig. 2 illustrate the intracellular uptake and staining of breast cancer cells with fluorescent nanozymes. The bright-field contrast image (Figure 2A) depicts the native shapes, morphology, and arrangement of untreated cells in a monolayer culture.

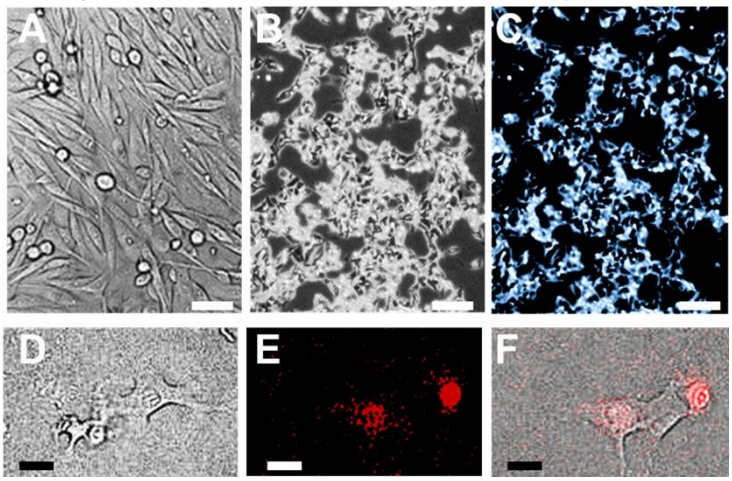

Figure 2. Confocal microscopy images of melanoma B16-F10 breast cancer cells. (A) Bright field contrast image of untreated cancer cells. (B) Epifluorescence image of cells stained with nanozyme. (C) Confocal microscopy image revealing the intracellular uptake of nanoparticles based on their one-photon fluorescence. (D) A bright-field contrast image of cancer cells treated with nanozyme. (E) Two-photon excited fluorescence imaging of the stained cells irradiated with 700 nm NIR. (F) Merged

micrograph showing the nanoparticle distribution in the intact cells based on the upconversion fluorescence.

Nanoparticle uptake and accumulation in the cytoplasm are evident in the epifluorescent image (Fig. 2B), based on one-photon (down-conversion) fluorescence emission. Further fluorescence microscopic observation revealed irreversible changes in cellular morphology (Fig. 2C), indicating the occurrence of a photo-induced cytotoxic effect caused by the nanozyme. An analogous bright-field contrast image of cancer cells treated with nanozyme is shown in Fig. 2D. Subsequently, cells were exposed to 700 nm NIR irradiation, and two-photon (upconversion) excited fluorescence emission was observed (Fig. 2E). The merged micrograph in Fig. 2F illustrates nanoparticle distribution within exposed intact cells, facilitated by emitted upconversion fluorescence. The impact of singlet oxygen production and induced ROS on melanoma cell migration was evaluated using an in vitro scratch assay under normoxic conditions. B16-F10 cancer cells were treated with photo-oxidase mimicking nanozymes under visible light irradiation, resulting in a significant decrease in migratory cells compared to controls. The nanozymes inhibited cell migration, achieving only 5–8% closure efficacy after 15 hours of treatment, suggesting potential control over metastatic migration when combined with near-infrared energy dosage. Biocompatibility assessments confirmed the safety of the nanozymes, with an effective therapeutic concentration of approximately 50 mg/l, tenfold lower than the threshold dose, and without influencing the signaling pathways of healthy cells.

► *Light-triggered Janus nanomotor*. Their distinct shape, charge properties, and the potential for coupling with other functional groups make them versatile tools for creating drug delivery systems and artificial therapeutic antibodies. A significant advantage lies in the ability of ultra-small gold nanoparticles capped with amino-modified oligonucleotides to form larger, functional self-assembled structures, enhancing their potential to navigate through leaky vasculature and accumulate at tumor sites. Furthermore, the specific snowflake-like supramolecular shape can be engineered by incorporating amine-modified oligonucleotides. Introducing additional functionality into the nanostructure design through nanoparticle packing within a nucleic acid shell could enable specific recognition of cancer cells and binding to their overexpressed receptors with high selectivity and affinity, facilitating active targeting within the target region.

► *Anisotropic nanosnowflakes*. The process of creating nanosnowflakes involved immobilizing double-stranded amine-modified and thiol-terminated DNA or RNA oligonucleotides onto the surface of ultra-small isotropic gold nanoparticles, acting as nanocarriers. The anisotropic nanosnowflakes hold promise as platforms for developing therapeutic materials and drug delivery systems. Their distinct shape, charge properties, size (40~80 nm), and the potential for coupling with other functional groups make them versatile tools for creating drug delivery systems and artificial therapeutic antibodies. The enhanced functionality of nanosnowflake design may improve recognition and binding to overexpressed receptors on cancer cells.

► *Effect of the theranostic nanomachines on the healthy human cells*. After exposure to theranostic nanoparticles for 6 hours and subsequent incubation for seven days, A549 and HepG2 cells showed minimal to no impact on cell proliferation. Growth curves indicated a gradual increase in cell number in both treated and untreated cultures, with no evidence of apoptotic or necrotic cells observed by the 6th day post-treatment. Cell morphology and shape remained unchanged, with homogeneous cytoplasm and no discernible nuclear or cytoplasmic alterations. Consistent with previous studies, the nanoparticles showed inability to penetrate the cell membrane, and they had negligible effects on the activity of DAO and ATPase in the absence of illumination. These findings suggest the nanoparticles' safety and limited impact on cellular function, supporting their potential for biomedical applications.

► *Future perspectives*. Theranostic nanomachines are being redesigned to enhance CAR-T therapy for cancer, with several potential applications under investigation. Targeted delivery involves engineering nanomachines to deliver CAR-T cells specifically to tumor sites, minimizing off-target effects. Nanomachines hold the potential to improve CAR-T therapy efficacy by overcoming barriers within the tumor microenvironment. They are also being developed as platforms for combination therapy, delivering CAR-T cells along with chemotherapy drugs, immune checkpoint inhibitors, or cytokines, to enhance the overall anti-cancer response and overcome resistance mechanisms. Ongoing research is exploring the full scope of these applications, aiming to optimize CAR-T therapy for improved cancer treatment outcomes.

５．主な発表論文等

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２．論文標題 Engineering of functional nanosnowflakes from gold nanocarriers capped with amino‐modified <scp>DNA</scp> oligonucleotides	５．発行年 2023年
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１．著者名 Machreki Manel、Chouki Takwa、Tyuliev Georgi、Zigon Dusan、Ohtani Bunsho、Loukanov Alexandre、Stefanov Plamen、Emin Saim	４．巻 8
２．論文標題 Defective Ti0₂ Nanotube Arrays for Efficient Photoelectrochemical Degradation of Organic Pollutants	５．発行年 2023年
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２．論文標題 Phytoremediation and extraction of colloidal contaminants from mine effluents through legume plants	５．発行年 2023年
３．雑誌名 Sustainable Extraction and Processing of Raw Materials Journal	６．最初と最後の頁 6～10
掲載論文のDOI（デジタルオブジェクト識別子） 10.58903/u17190823	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計1件（うち招待講演　1件／うち国際学会　1件）

１．発表者名 Alexandre Loukanov
２．発表標題 Light-triggered nanomachine for cancer theranostics
３．学会等名 XIII National Medical Physics and Biomedical Engineering Conference, NMPEC-2020（招待講演）（国際学会）
４．発表年 2020年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

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７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関