研　究　報　告　書

「がんの可視化と光線治療に向けた光分解性バイモーダルナノパーティクルの開発」

研究タイプ：通常型
研究期間：　平成 22 年 10 月～平成 26 年 3 月
研 究 者 ：Vasudevan Pillai Biju

1.　研究のねらい

Aim of this project is the efficient utilization of light for life innovation through the development of novel light-sensitive fluorescent- magnetic bimodal nanomaterials for bioimaging and phototherapy of cancer. Nanomaterials formulations, also called theranostics, with more than one modality for both biomedical imaging and therapy have become promising in the accurate screening and efficient treatment of diseases such as cancer. For example, nanomaterials such as silica nanoparticles, polymer nanoparticles, carbon nanotube, graphene, gold nanoparticles, liposomes, etc. are extensively investigated as theranostics or host materials for the incorporation of multiple contrast agents and drugs. These theranostics applied in animal models show excellent contrast enhancement in bioimaging modalities such as positron emission tomography (PET), X-ray computed tomography (CT), single-photon emission computed tomography (SPECT), magnetic resonance imaging (MRI) and fluorescence imaging, and improved curing of cancer under chemotherapy, photodynamic therapy (PDT) and photothermal therapy. Among the imaging modalities, MRI and fluorescence receive great attention in the scenario of increased health risks after biomedical imaging using ionizing radiations such as X-rays. Furthermore, the potential of fluorescence imaging modality to be combined with PDT is of particular interest in the management of cancers. However, as a result of the incorporation of multiple entities, the size of theranostics easily exceeds the limit of renal clearance, which poses a major limitation in their removal from biological systems. This project is aimed at lifting the above limitation by developing novel photouncaging theranostics and validating their photouncaging processes. This project is also aimed at the efficient use of photouncaging theranostics for bimodal (MRI and fluorescence) bioimaging *in vitro* and *in vivo* and PDT of cancer cells (Fig.1).

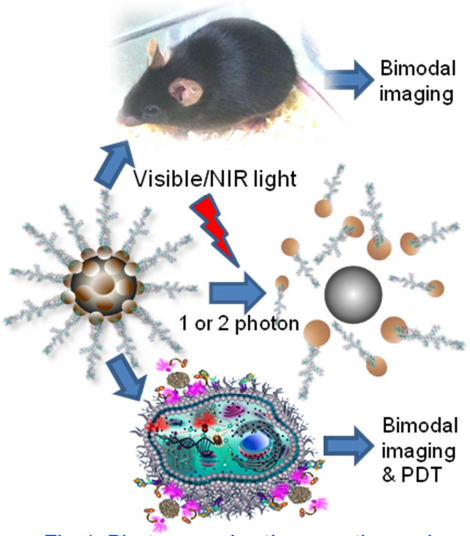

Fig.1: Photouncaging theranostics and their uncaging and applications aimed at

2.　研究成果

（1）概要

By using light as an innovative tool, herein this project introduces and evaluates the concept of photouncaging theranostics composed of fluorescent [gold quantum clusters (QCs), CdSe/ZnS quantum dots (QDs), porphyrin, etc.] and magnetic nanomaterials (Fe_3O_4, Gd complex, etc.) for bimodal imaging and photodynamic therapy. Chemical caging of fluorescent and magnetic entities into bimodal nanoparticles using photouncaging ligands, which is followed by the conjugation of intracellular delivery vehicles, allows us for the labeling of living cells and obtaining of MRI and fluorescence images. Selective one– or two–photon activation of the theranostics results in the systematic uncaging of the ligands and the nanoparticles. The potentials of photouncaging theranostics for *in vivo* imaging is validated by obtaining MRI and fluorescence images of mice subcutaneously or intravenously injected with the theranostics. Further, photouncaging theranostics composed of Au QCs or porphyrins efficiently produce singlet oxygen ($^1O_2$), which is the essential element for PDT. The renal clearance and phototherapeutic potentials of photouncaging theranostics are subjects of active research in our laboratory.

(2)詳細

研究テーマA「Chemical caging and photouncaging of theranostics」

At first, novel photouncaging and biocompatible molecules (1 and 2 in Fig. 2) composed of biotin and coumarin, which are the linkers of magnetic and fluorescent entities in theranostics. The steps involved in the synthesis of these molecules follow standard nucleophilic substitution and rearrangement reactions. The structures of these linker molecules are characterized using NMR, mass and IR spectroscopic techniques. Interestingly, as shown in Fig. 2A, these linkers efficiently uncage under one– or two–photon activation, which results in the systematic changes of UV–Vis absorption (Fig. 2B), mass and NMR spectra. Next, photouncaging theranostics are constructed by the conjugation of the linkers to streptavidin–functionalized super paramagnetic iron oxide (Fe_3O_4) nanoparticles (SPION) followed by the conjugation of multiple

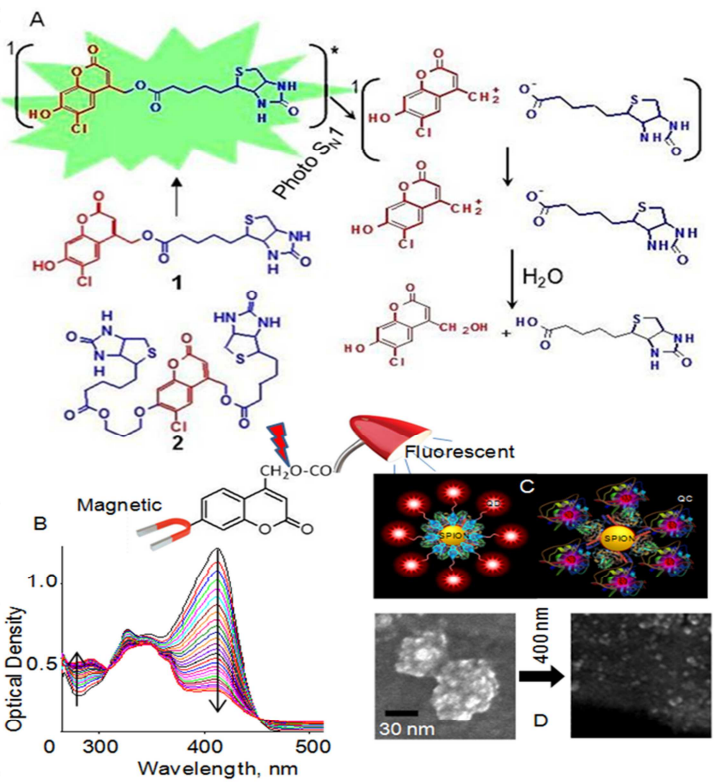

Fig.2: (A) Photouncaging process of **1** and **2**, (B) UV-Vis absorption spectra of 1 with time under photoactivation, and (C) the structures and (D) photouncaging of theranostics.

(ca 10) CdSe/ZnS QDs or Au QCs, which provided us with fluorescent–magnetic theranostics ($Fe_3O_4QD_{10}$ and $Fe_3O_4QC_{10}$, Fig.2C). Here, streptavidin–functionalized QDs are commercially obtained; whereas, Au QC and its streptavidin derivative are prepared in our laboratory. In these preparations, QDs (650 or 705 nm) and QCs (670 nm) with NIR emission are selected for enabling efficient bioimaging both in vitro and in vivo. Further, Au QCs produce $^1O_2$ in ca 15% efficiency, which is promising for the PDT application of theranostics; whereas, theranostics with high (ca70%) $^1O_2$ production efficiency are constructed by the preparation of novel porphyrin derivatives and their conjugation to SPION or $Fe_3O_4QD_{10}$. Two other fluorescent–magnetic bimodal nanoparticles prepared in this project are QDs (NIR fluorescent) conjugated with gadolinium (Gd^{3+}) complex (magnetic) and SPION (magnetic) conjugated with terbium (Tb^{3+}) complex (green fluorescent). Bimodal nature of the theranostics is characterized by recording MRI and fluorescence images of the samples. Finally, photouncaging of the theranostics is validated by recording and analyzing their scanning electron micrographs (Fig. 2D).

In short, novel magnetic, NIR fluorescent and $^1O_2$–producing theranostics are constructed using photouncaging ligands, and their photouncaging processes are validated.

研究テーマ B「Bimodal bioimaging using photouncaging theranostics」

The combination of MRI and fluorescence contrast agents in the photouncaging theranostics prepared under theme A allows us to use those for obtaining combined MRI and fluorescence images in vitro and in vivo. At first, the theranostics for bimodal imaging and cytotoxicity in cultured cancer cells of human or murine origin were tested. Hormones such as allatostatin or epidermal growth factor (EGF) were employed for the intracellular delivery of theranostics. The hormones are conjugated to the surface of photouncaging theranostics by following standard bioconjugate reactions. The cells are treated successively with the bioconjugated theranostics and the nucleus staining Syto dye. After the treatments, the cells are copiously washed with buffer and the medium is exchanged with the standard cell culture medium. The intracellular delivery of bioconjugated theranostics is investigated using fluorescence microscopy. Figs. 3A and 3B show the fluorescence images of murine melanoma (B16) and human lung epithelial adenocarcinoma (H1650) cells labeled with $Fe_3O_4QD_{10}$ and $Fe_3O_4QC_{10}$, respectively. The NIR fluorescence of QDs and QCs allows us for obtaining fluorescence

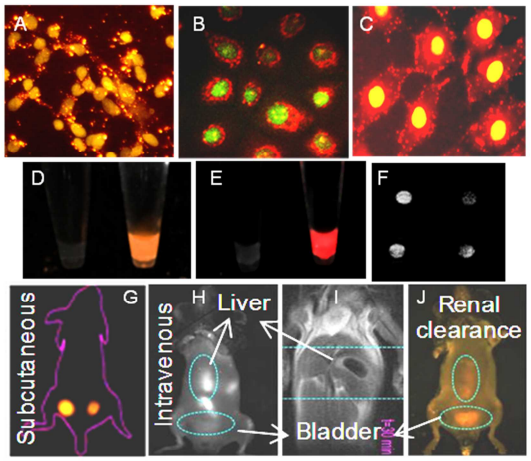

Fig.3: Fluorescence images of (A,D) B16 cells/cell pellet labeled with $Fe_3O_4QD_{10}$,(B,E) H1650 cells/cell pellet labeled with $Fe_3O_4QC_{10}$, (C) H1650 cells labeled with QDs, (F) MRI of cell pellets, (G-J) fluorescence/MRI images of B6 mice injected with $Fe_3O_4QD_{10}$: (G) subcutaneous, (H-J) intravenous.

images of cells without any interference of cell autofluorescence. Further, the cells are harvested by tripsinization into cell pellets and recorded the MRI (Fig. 3F) and fluorescence (Fig. 3D,E) images. The enhanced MRI contrast of cells, which is due to the huge magnetic dipole moment of Fe_3O_4 when compared with that of water and biomolecules in cells, is a promising property that can be combined with NIR fluorescence for in vivo bimodal bioimaging. The amounts of theranostics needed for the labeling and fluorescent–magnetic bimodal imaging of cells are in the 0.5 to 2 nM regime, which is far below the toxic levels (>>100 nM) to cells, are evaluated using standard cytotoxicity assays.

The potentials of the theranostics for in vivo bimodal imaging are validated by the subcutaneous or intravenous injection in B6 mice, which is followed by the the MRI and NIR fluorescence imaging (Fig. 3G–J). The NIR fluorescence and MRI contrast of the theranostics enabled us for the efficient detection of the biodistribution, accumulation in the liver and renal clearance of the particles. The renal clearance is also validated by the analysis of fluorescence and MRI contrasts of urine samples.

In summary, theranostics are successfully delivered in living cancer cells or injected in mice, and the NIR fluorescence and MRI contrast enhancements enabled us for validating the potentials of the theranostics for in vitro and in vivo bimodal bioimaging with excellent signal to noise ratios.

研究テーマ C「Theranostics for singlet oxygen production and photodynamic therapy」

Theranostics composed of QCs produce $^1O_2$ in ca 15% efficiency, which is characterized by recording the time–resolved fluorescence spectra of QCs in the presence and absence of oxygen and under different excitation power density. Considerable increase of QC's fluorescence lifetime (Fig. 4A) with time under nitrogen gas purging and decrease of fluorescence lifetime with increase of excitation power suggest the quenching of the excited state of QCs by oxygen and the production of $^1O_2$. This photosensitized production of $^1O_2$ is further confirmed by using a $^1O_2$ sensor dye molecule as well as by recording the characteristic (1270 nm) chemiluminescence spectrum of $^1O_2$ (Fig. 4B). On the other hand, the efficiency of $^1O_2$ production by QD–based theranostics is negligibly low, which is considerably improved by the conjugation of newly prepared porphyrin derivatives to either SPION or $Fe_3O_4QD_{10}$.

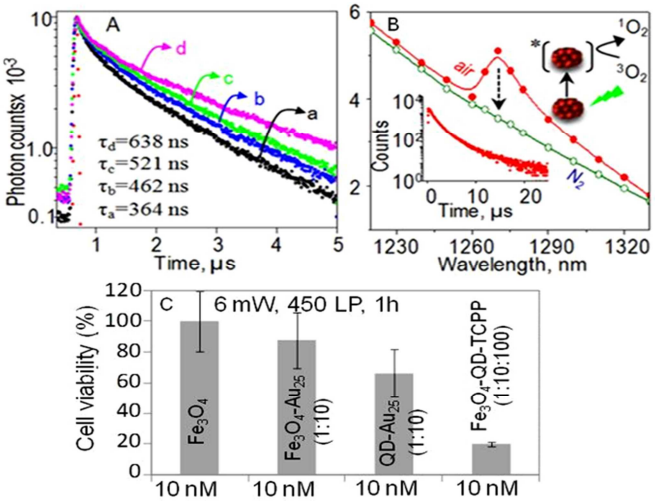

Fig.4: (A) Fluorescence decay profiles of QCs with elapse (a to d) of time under nitrogen purging. (B) Chemiluminescence spectra of $^1O_2$ produced by QCs under air saturated (red) and nitrogen saturated (green) conditions. Inset: decay at 1270 nm. (C) Histograms of MTT assay for H1650 cells labeled with different samples and photoactivated.

The production of $^1O_2$ by QC- or porphyrin- conjugated theranostics is a promising property for PDT, which is evaluated in H1650 cells cultured in 96 well plates. Here, the cells are labeled with 1 to 100 nM $Fe_3O_4QC_{10}$, or porphyrin-conjugated Fe_3O_4 or $Fe_3O_4QD_{10}$. Successively, the cells are washed and photoactivated for 30 min to 1h under 450 nm long-pass-filtered light (6 mW/cm^2) from a Xe-lamp. After the photoactivation, the cells are treated with the cell permeable 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Viable cells convert yellow MTT into purple formazan as a result of the NAD(P)H-dependent cellular oxidoreductase enzymes. After 4h incubation of the photoactivated cells with MTT and the subsequent lysis of cells, the amount of formazan produced, which is the direct measure of cell viability, is measured using a microplate reader (Fig. 4C,D). Remarkable reduction in the viability is observed for cells treated with porphyrin-conjugated Fe_3O_4 or $Fe_3O_4QD_{10}$ and photoactivated.

The production of $^1O_2$ by theranostics and the associated reduction in the viability of cultured cancer cells show the potentials of theranostics for practical PDT application.

3. 今後の展開

The new concept of photouncaging theranostics validated at an interface among light, nanomaterials, bioimaging and photodynamic therapy in this project is expected to untangle challenging issues in life science by the nucleation of new research projects in different directions. The most promising direction is the in vivo application of theranostics for human use such as imaging, phototherapy, and clearance by uncaging using light of suitable energy. In such applications, the photouncaging properties can be combined with photo-controlled delivery of drugs, genes, and contrast agents. Further, photouncaging ligands and nanoparticles can be applied to microfabricated devices for the collection of cancer cells from blood samples as well as the collection, separation and characterization of pathogens. Yet another promising aspect of photouncaging nanoparticles is their extension towards the construction of reusable photoresponsive micro and nano platforms for the collection and detection of biomarkers and exosomes.

4. 評価

By combining NIR fluorescent and magnetic nanomaterials with singlet oxygen production using photouncaging ligands, the project successfully proves the concept of photouncaging fluorescent-magnetic theranostics. In addition to the validation of bimodal bioimaging and photodynamic therapy, the photouncaging strategy in this project successfully lifts one of the major challenges and concerns associated with the use of large-size theranostics in biological systems. Following the evaluation of the bimodal nature and photouncaging, conjugation of biomolecules to the photouncaging theranostics opened up in vitro and in vivo MRI and fluorescence imaging potentials, which is validated in cells and mice. The choice of gold quantum clusters not only provides non-toxic nanomaterials and NIR fluorescence but also singlet oxygen for photodynamic therapy. The limitation associated with the low efficiency of singlet oxygen

production by gold clusters is lifted by the preparation porphyrin conjugated theranostics. Overall, the project is evaluated as the successful validation of photouncaging theranostics for bimodal imaging and phototherapy with its doors opened for practical applications such as drug delivery, phototherapy, biomedical imaging, and the construction of reusable micro and nano platforms for the detection of cancer cells, pathogens and biomarkers.

（2）研究総括評価（本研究課題について、研究期間中に実施された、年2回の領域会議での評価フィードバックを踏まえつつ、以下の通り、事後評価を行った）。

X 線のような放射線治療は放射線障害による健康リスクが問題となるため、磁場や低出力レーザーを用いた MRI・蛍光イメージング法と光線療法に関心が集まってきた。結果として、蛍光ナノ粒子、MRI 造影剤や光線療法薬剤はがんの検出・治療のための治療的診断法用として広く研究が行われているが、既存のナノ粒子や薬剤はそのサイズの大きさのため生体器官への蓄積が問題となっている。

この問題を解決するためビジュ研究者は、蛍光・MRI イメージング両方に使用できるバイモーダル造影剤として、新規の無毒性かつ光分解可能なナノ粒子を開発した。このナノ粒子は光照射によって分解・断片化して尿により排泄されるため、生体器官に蓄積されてしまう問題を解決した。さらに、がん細胞やマウスのバイモーダルイメージを得るだけでなく、光線力学的療法に有用な一重項酸素の産生をも可能にした。このような光分解ナノ粒子は、薬剤送達・遺伝子の制御やがん細胞の検出・イメージング、そしてがん細胞の画像誘導温熱療法・光線療法への候補物質となることが見込まれる。

広く認識されている課題に果敢に挑戦し、非常にアクティブに研究活動を行ってきたことは、多くの論文などのアウトプットからも明らかである。さらに、使用後に光照射により分解して尿中に排出できるバイモーダル造影剤の開発に成功した事は、この分野に新しい概念を提供した事になる。新しい可能性を示す事は、他の研究者の参入を促し、この分野の研究活動が活発化する。まさに「さきがけ」研究らしい研究活動であったと評価している。

5.　主な研究成果リスト

（1）論文（原著論文）発表

1. V. Biju, “Chemical modifications and bioconjugate reactions of nanomaterials for sensing, imaging, drug delivery and therapy”, *Chem. Soc. Rev.* 43, 744–764 (2014).
2. E. S. Shibu, S. Sugino, K. Ono, H. Saito, A. Nishioka, S. Yamamura, M. Sawada, Y. Nosaka, V. Biju, “Singlet-oxygen-sensitizing near-infrared-fluorescent multimodal nanoparticles”, *Angew. Chem. Int. Ed.*, 52, 10559–10563 (2013).
3. E. S. Shibu, K. Ono, S. Sugino, A. Nishioka, A. Yasuda, Y. Shigeri, S. Wakida, M. Sawada, V. Biju, “Photouncaging nanoparticles for MRI and fluorescence imaging in vitro and in vivo”, *ACS Nano,* 7, 9881–9859 (2013).
4. P. Jones, S. Sugino, S. Yamamura, F. Lacy, V. Biju, “Impairments of cells and genomic DNA by environmentally transformed engineered nanomaterials”, *Nanoscale*, 5,　9511–9516, (2013).
5. E. S. Shibu, M. Hamada, N. Murase, V. Biju, “Nanomaterials formulations for photothermal and photodynamic therapy of cancer”, *J. Photochem. Photobiol. C: Photochem. Rev.*, 15, 53–72 (2013).

6. V. Biju, A. Anas, H. Akita, E. S. Shibu, T.Itoh, H. Harashima, M. Ishikawa, "FRET from quantum dots to photodecompose undesired acceptors and report the condensation and decondensation of plasmid DNA", *ACS Nano,* 6, 3776–3788 (2012).

(2)特許出願

研究期間累積件数:1 件

(平成 26 年 3 月時点で非公開)

(3)その他の成果(主要な学会発表、受賞、著作物、プレスリリース等)

＜学会発表＞

1. Biju, V. (plenary), "Engineered nanomaterials: Potential agents for biological applications or potentiators of toxicity" Embassy of India, Tokyo, October 11th 2013.
2. Biju, V. (invited), "Nanomaterials formulations for bioimaging", Nanotech 2014, Tokyo Big Sight, January 30th 2014.
3. Biju, V. (invited), "Nanomaterials for Multiplexed Fluorescence Imaging", the 4th Asian Spectroscopy Conference, Nanyang Technological University, Singapore, December 17th 2013.
4. Biju, V. (invited), "Multifunctional engineered nanomaterials: Bioimaging applications vs toxicity", International Workshop on Photonics of Functional Nanomaterials, City University of Hong Kong, 9th May 2013.
5. Biju, V. (invited), "Engineered Nanomaterials for Advanced Bioimaging", International Conference on Materials Science ICMAT 2013, Singapore, 4th July 2013.
6. Biju, V. "Photoresponsive nanomaterials for bioimaging", International Conference on Photochemistry 2013, Leuven, Belgium, 24th July 2013.
7. Biju, V.; Shibu, E. S.; Sugino, S.; Yamamura, S.; Wakida, S.; Saito, H.; Nosaka, Y.; Ono. K.; Sawada, M. "Photouncaging Nanoparticles for Bioimaging", 2013 Annual Meeting of the Japanese Photochemistry Association, Ehime University, Matsuyama, Japan, 13th September 2013.

＜受賞＞

1. 「Asian and Oceanian Photochemistry Award for young scientists」(2010)
2. 「光化学協会奨励賞」(2011)
3. 「英国王立化学会フェロー」(2011～)