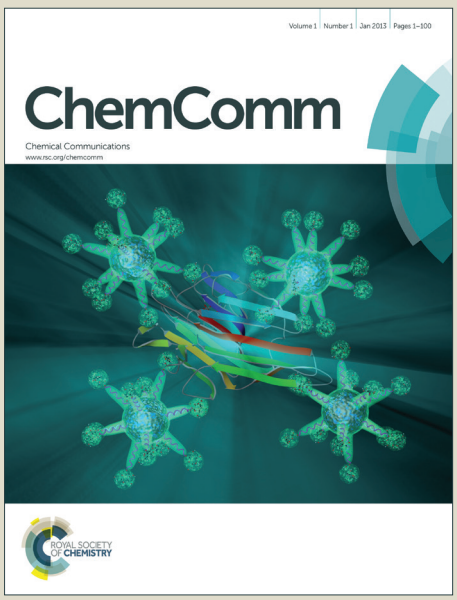
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

COMMUNICATION

Photoinduced cytotoxicity of a photochromic diarylethene via caspase cascade activation

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

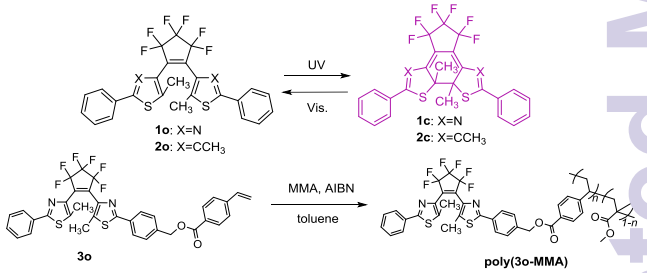
www.rsc.org/

Jun-ya Okuda,[a] Yukimi Tanaka,[a] Ryuhei Kodama,[a] Kimio Sumaru,*[b] Kana Morishita,[b] Toshiyuki Kanamori.[b] Seiji Yamazoe,[a,c] Kengo Hyodo,[a] Shohei Yamazaki,[a] Tomohiro Miyatake,[a] Satoshi Yokojima,[d,e] Sinichiro Nakamura,[e] and Kingo Uchida*[a]

The photo-generated closed-ring isomer of bis(5-methyl-2-phenylthiazoyl)perfluorocyclopentene shows cytotoxicity to Madin-Darby canine kidney (MDCK) cells through caspase cascade and induces apoptosis of the cells.

Diarylethenes are photoswitchable molecules and they have been applied to optical memory and devices.[1-3] Although their biological applications have been few, the use of photoswitches in this area has recently attracted growing interest.[4-6] The technology of "on-demand killing of adherent cells" is important as well as selective cell adhesion technique. Photoresponsive molecules such as photochromic spiropyran derivatives and photo-acid generator molecules have been used for such applications.[7,8] Diarylethenes are also used in this field. Branda *et al*. demonstrated that a photoresponsive system can reversibly induce paralysis in nematodes as a model for the living organisms *Caenorhabditis elegans* when two different wavelengths of light are used to toggle the diarylethene molecular switch between its two structural forms.[9] Furthermore, Yi *et al* demonstrated that another diarylethene works as the photoswitchable probe for imaging living cells.[10] Feringa proposed a new concept called "photopharmacology" in which the drug activity in time and space is controlled by the use of light as an external control element.[11]

Previously, we studied the photocontrol of microcrystalline surfaces with topographical changes.[12,13] During the study we found by chance that one of the diarylethene derivative generated SO_2 gas accompanied with decomposition by UV irradiation, thus inducing cell death.[14] On the other hand, on the film of diarylethene derivative **1o**, which shows high fatigue resistance without decomposition,[15] we also found a photoinduced cell death upon UV irradiation by a different mechanism. In this study, we examined the photo-toxicity of the diarylethene photoswitch itself upon light irradiation.



Scheme 1. Photochromic reaction of diarylethenes **1o, 2o,** and preparation of a photochromic polymer **poly(3o-MMA).**

As reported in our previous paper, diarylethene **1o** showed photochromism in organic solvents,[16] and diarylethene derivative **1o** also showed photochromism even in solvents containing a large amount of water. Figure 1 shows the spectral changes of **1o** in a mixed solvent consisting of ethanol and water (7:3 v/v).

Although the solubility of **1o** in an aqueous system was very low (thus the concentration of the sample solution was much smaller), λ_{max} values for both isomers were nearly the same as those in an ethanol-water mixture.

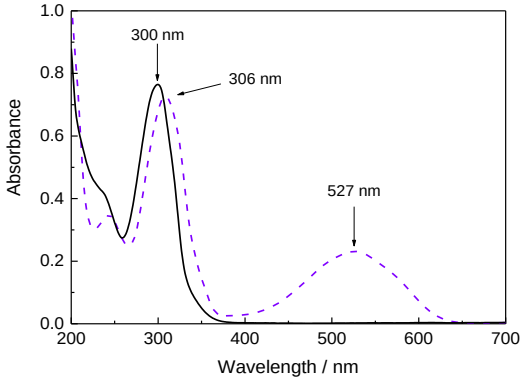


Fig. 1 Absorption spectral changes of diarylethene **1o** in a mixed solvent of ethanol and water (7:3 v/v). **1o** before UV irradiation: solid line, photostationary state (**1o:1c** ~ 0:100) upon 313 nm light irradiation: broken line.

[a] Address here.
[b] Address here.
[c] Address here.
† Footnotes relating to the title and/or authors should appear here.
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

The conversion to closed-ring isomer **1c** at the photostationary states upon UV light irradiation of 313 nm was estimated to be 100%, using the absorption coefficients in hexane. We also observed that the absorption spectra returned to the original spectrum by ring opening (cycloreversion) upon irradiation with visible light (wavelengths: 436 or 546 nm).

On a surface coated with diarylethene **1c** at a density of 0.6 μg/cm^2, MDCK cells were disseminated and cultivated for 1 day (Fig. 2a). In response to the blue light (436 nm, 140 mW/cm^2) irradiation on the patterned area ("436") for 2 min (Fig. 2b), the cells detached (Fig. 2c). We observed that most of the cells in the irradiated area were dead upon light irradiation of 436 nm. We also examined the influence of the green light (λ= 546 nm), which induces ring opening of **1c**. No detectable change was observed upon 546-nm light irradiation even at a dosage that was more than doubled. By contrast, 436-nm light irradiation resulted in apparent cell damage.

In the case of using **1o** instead of **1c**, on the other hand, such cell damage was induced neither by 365-nm light irradiation which induces ring closure of **1o,** nor by 436-nm light irradiation. Therefore, the 436-nm light was lethal for **1c**-dosed cells. More interestingly, no detectable cell damage was observed even in the case where 436-nm light was irradiated just after-365 nm light irradiation which can isomerize **1o** to **1c**. This result suggested that it takes time for **1c** to exert a lethal effect in response to 436-nm light irradiation. This can be achieved through the structural rearrangement in the cell environment.

In an attempt to clarify the effect of molecular diffusion on the cell damage, we examined the immobilization of diarylethene at the side chain of water-insoluble polymer and thus the influence of the 436-nm-irradiated diarylethene from outside the cell.

Water insoluble polymer **poly(3o-MMA)** which contains diarylethene as the pendant groups, was prepared by the radical co-polymerization of monomer **3o** and MMA with an AIBN initiator. In order to obtain **poly(3c-MMA)**, the culture substrate coated with **poly(3o-MMA)** was irradiated with UV light (254 nm) until it reached photo-stationary state (PSS). Due to the high cyclization quantum yield (0.37) and low cycloreversion quantum yield (0.02), the PSS was dominated by a **poly(3c-MMA)** state(Fig. S1).[14] Although the areal density of diarylethene was much larger (> 10 times) than the case using monomeric **1c**, the same dosage of 246 nm of UV light irradiation did not result in observable cell damage. The cells in the photoirradiated area remained as well as those in the non-irradiated area (Fig. S2). This means that fixed and insoluble diarylethene moieties did not show any cytotoxicity to the cells. These results indicate that monomeric diarylethene is essential to achieving photo-induced cell death. Thus, **1c** was most likely delivered to specific site in the cell, and then exerted a lethal effect in response to 436 nm light irradiation.

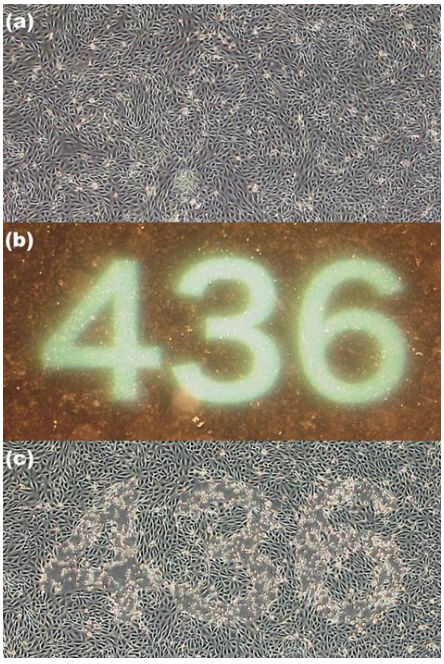


Fig. 2 MDCK cell damage on a **1c**-coated surface. (a) before light irradiation, (b) during irradiation and (c) after irradiation with 436 nm light (140 mW/cm^2).

Here we summarize the results of cell experiments using MDCK cells with respect to the conditions of diarylethene dosage and light irradiation:

(1) Photo-induced cell death was observed when 436-nm light was irradiated to **1c**-dosed cells.
(2) The death of **1c**-dosed cells was not induced by 546-nm light irradiation.
(3) For **1o**-dosed cells, 436-nm light irradiation was not lethal even immediately after 365-nm light irradiation to induce photo-isomerization of **1o** to **1c**.
(4) On **poly(3c-MMA)** as immobilized **1c**, photo-induced cell death was not observed.

These results imply that only the diarylethene in the **1c** state reached some critical site in the cell (i.e. DNA or mitochondria), at which the diarylethene would be ready to exert a lethal effect in response to light irradiation (Fig. 3a). Once the monomeric diarylethene integrated in the critical site, regardless of whether in **1c** form or in **1o** form, the cell could be damaged upon 436-nm light irradiation (Fig. 3b). On the other hand, result (3) suggests that molecule **1o** was not integrated in the critical site (Fig. 3a, 3c). Furthermore, result (4) implies that there was no photo-induced lethal effect from the diarylethene immobilized on a culture surface close beneath but outside the cultured cells (Fig. 3d). In the following two subsections, the interaction of the monomeric diarylethene with DNA and its membrane disrupting property are examined to investigate the elementary steps to cell death. Finally, we discuss the generation of the reactive oxygen species as a possible photo-toxicity brought about by 436-nm light irradiation.

Many compounds known to be good intercalators of DNA have planer structures. Considering that **1c** and **1o** have planer and non-planar structures, respectively, some photo-induced action of **1c** to DNA can be viewed as one of the possible mechanisms of observed cell death. Therefore, we examined the interaction between **1c** and DNA by measuring the change in its absorption spectra; specifically, we investigated the absorption spectra of **1c** in phosphate buffered saline (PBS) under several conditions of DNA coexistence after dissolution of **1c** and 15-hour incubation at 40 ˚C. The absorbance of DNA was negligible compared with that of **1c** in the wavelength range 350-750 nm. Since the dissolved state of **1c** in aqueous systems was not stable due to its hydrophobicity, without DNA the absorbance decreased 15 hours after the dissolution of **1c** into PBS through their association or precipitation. In cases of the coexistence of DNA, on the other hand, the decrease was

suppressed effectively and we observed absorption maximum typical for **1c** (Fig. S3). This result suggests that the coexisting DNA stabilized **1c** that was dissolved in aqueous systems. Furthermore, native DNA showed a greater stabilization effect than the decomposed DNA by deep UV light (wavelength: 254 nm) irradiation (Fig. S3). Considering its planar and hydrophobic structure, diarylethene in **1c** form was assumed to gain its stability by intercalating into DNA. This viewpoint is consistent with the results of cell experiments suggesting that only **1c,** which has such a planer structure, is delivered to a specific site in the cell.

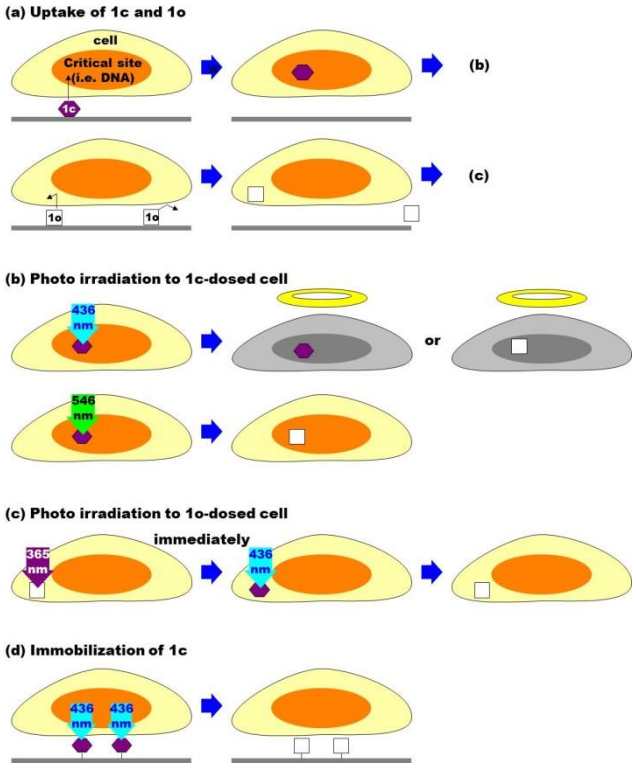


Fig. 3 Photo-induced effect of diarylethene to MDCK cells. Purple hexagon and open-square show **1c** and **1o**, respectively.

Very recently, P. Gamez *et al.*, reported the preparation of platinum complexes from photoswitchable 1,2-dithienylethene-containing ligands and the closed-ring isomer of the derivatives exhibiting DNA-interacting properties and cytotoxic behaviors.[17]

In this work, we observed the potential interactions between DNA and the open- and closed-ring isomers of **1** and **2** by a fluorescence reduction experiment based on the competitive binding of ethidium bromide (EB) and diarylethenes to the DNA. Displacement of EB from the fluorescent EB-DNA adduct by a DNA-interacting molecule will induce fluorescence quenching.[18] Fluorescence spectra were monitored at constant concentration of DNA and EB (15 and 75 μM, respectively), while adding increasing amount of **1** and **2** (in the range of 5-25 μM). A clear decrease in emission intensity was observed only for **1c** as shown in the S. I. (Fig. S4) and the affinity of the open- and closed-ring isomers for DNA was evaluated and compared using the Stern-Volmer quenching constant, K_{SV}. The K_{SV} values for **1o**, **1c**, **2o**, and **2c** are 6.0×10^2, 7.6×10^3, 7.0×10^2, and 1.4×10^3 M^{-1}, respectively. The results agreed

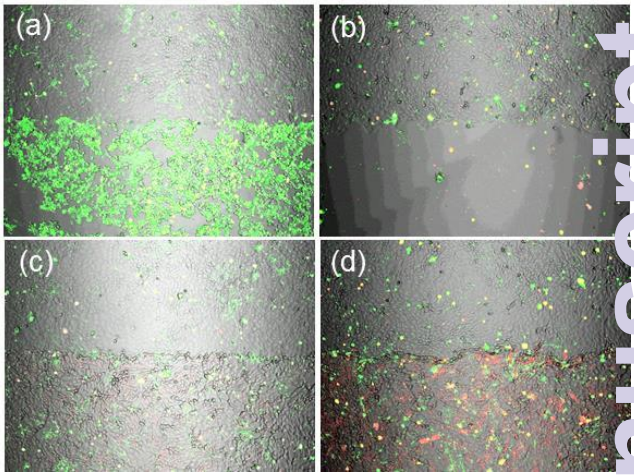


Fig. 4 Apoptosis assay of the MDCK cells dosed with 0.2 ppm of **1c** i the absence (a, b) or presence (c, d) of caspase inhibitor Z-VAD-FMI respectively. The cells were stained with Apoptotic & Necrotic Cell Detection Kit and observed 2 (a, c) or 4 (b, d) hours after irradiation, respectively, in the lower half part (upper half part is n irradaited area). Green and red fluorescence were from exteriorized phosphatidylserine exposed on the cell surface, and from the nuclei of the cells having leaky cell membrane, respectively.

well with the results showing that only **1c** but not **1o, 2o, 2c** showed cytotoxicity.

In considering the mechanism of the cytotoxic expression mentioned above, an important clue may be given by an analysis o the types of cell death. It is known that the cells which suffer fat damage on an intrinsically vital part, such as DNA and mitochondri go to their deaths actively through the apoptotic process by mainl caspase cascade, even if the cells avoided instant death (necrosis, Figure 4 shows the results of apoptotic assay of MDCK cells upo being partially irradiated for 2 min with blue light (436 nm, 8 mW/cm^2) after being cultured with an addition of 0.2 ppm of **1c**. B dyeing with the Apoptotic & Necrotic Cell Detection Kit two hou (Figs. 4a, c) or four hours (Figs. 4b, d) after the blue light irradiation, exposed phosphatidylserine (PS) outside of a cell membrane (a n of apoptosis) was marked green by annexin V labeled with Fluorescein isothiocyanate (FITC)(Fig. S5), whereas the nucleus of the cell which lost the soundness of the cell membrane was marke red by ethidium homodimer III. Two hours after the blue ligh irradiation, in the absence of Z-VAD-FMK, caspase inhibitor, majority of the cells emitted green fluorescence and a very sma number of the cells emitted red fluorescence (Fig. 4a). These result show the exteriorization of PS in the cell membrane of which th soundness was maintained to some extent, and they are consistent with the result of membrane disruption assay. Four hours after th blue light irradiation, most cells were detached from the irradiation area indicating the viability of the cells was greatly damaged ig. 4b). In contrast, as for the cells in the presence of Z-VAD-FMK, fluorescence from the cells two hours after irradiation with blue light was similar to that from the cells without irradiation (Fig. 4c but the domain of the red fluorescence was shown slightly dominant rather than that of the green fluorescence after four hours. The results indicate that the necrotic process gradually progressed during the apoptotic process due to caspase being restrained by Z-VAD-FMK. The results of these studies strongly suggest that the activation of cytotoxicity in response to irradiation

of blue light is induced by the direct action of a very small amount of **1c** to DNA or mitochondria, which are critical cites of a cell. In particular, the finding demonstrate the activation of the caspase cascade (green arrows) as shown in the S. I. (Fig. S5).

Conclusions

We have described the influence of the photoirradiation to the viability of cultured MDCK cell dosed with photochromic diarylethene bis(5-methyl-2-phenylthiazoyl)perfluorocyclopentene. Drastic cytotoxicity was observed to appear when the light of 436 nm wavelength was irradiated to the cells dosed with the small amount of diarylethene in the closed-ring isomeric state **1c**, while no photoinduced toxicity was observed for open-ring isomeric state **1o** and polymeric immobilized states of **1c** as the pendant groups. Further, the light of 546 nm wavelength, which could induce ring-opening of **1c**, did not cause any detectable cell damage. The UV-Vis spectral analysis carried out for **1c** in several coexistence condition of DNA the aqueous solution suggested that monomeric **1c** was integrated in the DNA of the cell, in which the diarylethene would be ready to exert cytotoxicity in response to light irradiation. Also apoptosis assay carried out for the **1c**-dosed MDCK cells after light irradiation suggested that very small amount of diarylethene provided DNA or mitochondria, critical cites of a cell, with the fatal effect in response to light irradiation. In the photodynamic therapy (PDT), phototoxicity remaining after the therapy is a problem occasionally. Suffering this problem, the patients must avoid to exposure the sunlight for considerable time. Therefore, the shortening of the time is an important research subject.[19] The photoswitching of phototoxicity shown in this study suggested a new scheme to solve the problem in a feasible way.

The authors are also grateful to Zeon Co., Ltd., for providing the octafluorocyclopentene to synthesize the compound **1o**, and Y. Issiki and S. Takeda for the preparation of a photochromic polymer **poly(2o-MMA).** This work was supported by the MEXT-Supported Program for the Strategic Research Foundation at Private Universities and a Grant-in-Aid for Scientific Research on Innovative Areas "Photosynergetics (No. 2606)" from MEXT, Japan. The cell experiment in this work was supported by JSPS KAKENHI (Grant-in-Aid for Scientific Research (B)) Grant Number 25282148.

Notes and references

[a] *Department of Materials Chemistry, Faculty of Science and Technology, Ryukoku University, Seta, Otsu, Shiga 520-2194, Japan, Fax: +81-77-543-7483; Tel: +81-77-543-7462; E-mail; uchida@rins.ryukoku.ac.jp*

[b] *Biotechnology Research Institute for Drug Discovery, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 5th, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, Japan*

[c] *Department of Chemistry, School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

[d] *School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*

[e] *RIKEN Research Cluster for Innovation, Nakamura Laboratory, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan*

† Electronic Supplementary Information (ESI) available: [Experimental Section, Absorption spectral changes of **poly(3o-MMA)** in acetonitrile solution, MDCK cells on copolymer **poly(3c-MMA)** after blue light irradiation, Absorption spectra of **1c** in PBS in several conditions of DNA coexistence, Stern-Volmer Plots of I_0 / I vs. concentrations of diarylethenes for the titration of DNA-EB with **1** and **2,** Stern-Volmer quenching constant, Schematic diagram of programmed cell death: apoptosis, and Photoinduced cytotoxicity of **2c** and **2o**. See DOI: 10.1039/b000000x/

References

1 M. Irie, *Chem. Rev.*, 2000, **100**, 1685–1716.
2 B. L. Feringa and W. B. Browne, Molecular Switches, Wiley-VCH, Weinheim, 2011.
3 K. Matsuda and M. Irie, *J. Photochem. Photobiol. C: Photochem. Rev.,* 2004, **5**, 169-182.
4 W. Szymanski, J. M. Beierle, H. A. V. Kistemaker, W. A. Velema, B. L. Feringa, *Chem. Rev*., 2013, **113**, 6114-6178.
5 P. Gorostiza and E. Y. Isacoff, *Science,* 2008, **322**, 395-399.
6 W. A. Velema, J. P. van der Berg, M. J. Hansen, W. Szymanski, A. J. M. Driessen, B. L. Feringa, *Nat. Chem.*, 2013, **5**, 924-928.
7 J. Edahiro, K. Sumaru, Y. Tada, K. Ohi, T. Takagi, M. Kameda, T. Shinbo, T. Kanamori and Y. Yoshimi, *Biomacromolecules*, **2005**, 6, 970-974.
8 K. Sumaru, K. Kikuchi, T. Takagi, M. Yamaguchi, T. Satoh, T. Morishita and T. Kanamori, *Biotechnol. Bioeng*., 2013, **110**, 348-352.
9 U. Al-Atar, R. Fernandes, B. Johnsen, D. Baillie and N. R. Branda, *J. Am. Chem. Soc.,* 2009, **131**, 15966-15967.
10 Y. Zou, T. Yi, S. Xiao, F. Li, C. Li, X. Gao, J. Wu, M. Yu and C. Huang, *J. Am. Chem. Soc,* 2008, **130**, 15750–15751.
11 W. A. Velema, W. Szymanski and B. L. Feringa, *J. Am. Chem. Soc.,* 2014, **136**, 2178–2191.
12 N. Nishikawa, A. Uyama, T. Kamitanaka, H. Mayama, Y. Kojima, S. Yokojima, S. Nakamura, K. Tsujii and K. Uchida, *Chem. - Asian J*., 2011, **6**, 2400-2406.
13 A. Uyama, S. Yamazoe, S. Shigematsu, M. Morimoto, S. Yokojima, H. Mayama, Y. Kojima, S. Nakamura and K. Uchida, *Langmuir*, 2011, **27**, 6395-6400.
14 R. Kodama, K. Sumaru, K. Morishita, T. Kanamori, K. Hyodo, T. Kamitanaka, M. Morimoto, S. Yokojima, S. Nakamura and K. Uchida, *Chem. Commun*., 2015, **51**, 1736-1738.
15 K. Uchida, T. Ishikawa, M. Takeshita and M. Irie, *Tetrahedron*, 1998, **54**, 6627-6638.
16 H. Nishi, T. Namari and S. Kobatake, *J. Mater. Chem.*, 2011, **21**, 17249-17258.
17 A. Presa, R. F. Brissos, A. B. Caballero, I. Borilovic, L. Korrodi Gregorio, R. Perez-Tomas, O. Roubeau, P. Gamez, *Angew. Chem. Int. Ed.,* 2015, **54**, 4561-4565.
18 F. J. Meyer-Almes, D. Porschke, *Biochemistry*, 1993, **32**, 4246-4253.
19 A. B. Ormond and H. S. Freeman, *Materials*, 2013, **6**, 817-840.