Bioorganic & Medicinal Chemistry 17 (2009) 3775–3781

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Preparation and fluorescence properties of fluorophore-labeled avidin–biotin system immobilized on Fe_3O_4 nanoparticles through functional indolequinone linker

Nao Hirata [a], Kazuhito Tanabe [a,*], Asako Narita [b], Kazuo Tanaka [b], Kensuke Naka [c], Yoshiki Chujo [b], Sei-ichi Nishimoto [a,*]

[a] Department of Energy and Hydrocarbon Chemistry, Graduate School of Engineering, Kyoto University, Katsura Campus, Kyoto 615-8510, Japan
[b] Department of Polymer Chemistry, Graduate School of Engineering, Kyoto University, Katsura Campus, Kyoto 615-8510, Japan
[c] Department of Chemistry and Materials Technology, Kyoto Institute of Technology, Matsugasaki, Kyoto 606-8585, Japan

ARTICLE INFO

Article history:
Received 6 April 2009
Revised 22 April 2009
Accepted 23 April 2009
Available online 3 May 2009

Keywords:
Chemical biology
Molecular imaging
Hypoxia
Magnetic nanoparticles
Indolequinone

ABSTRACT

We prepared and characterized a new class of fluorophore-labeled magnetic nanoparticles (MNPs) possessing a hypoxia-responsive unit to construct a hypoxia-selective emission system. The indolequinone derivative as a hypoxia-response unit bearing biotin was synthesized and immobilized on Fe_3O_4 MNP. Subsequent complexation of this functionalized MNP with fluorescein-labeled avidin formed fluorophore-labeled nanoparticles (**AF-QB@MNP**). The fluorescence intensity of **AF-QB@MNP** was suppressed because of the adjacent quenching function of the indolequinone moiety and MNP. Upon hypoxic treatment by NADPH:cytochrome P450 reductase, **AF-QB@MNP** was activated to liberate a fluorescence unit, leading to the significant enhancement of fluorescence emission, while a smaller enhancement in fluorescence emission occurred upon aerobic treatment. The **AF-QB@MNP** has a indispensable properties as a fluorescent probe for imaging of disease relevant hypoxic microenvironments.

1. Introduction

High-functionalized magnetic nanoparticles (MNPs) bearing inherent optical properties provide a new class of diagnostic agents.[1–5] MNPs have the following unique characteristics: first, MNPs can be readily modified by various functional groups;[6] second, MNPs quench the fluorescence emission of adjacent fluorophores mainly because of the strong absorption cross section of MNPs and to a lesser extent the dynamic and static quenching of the fluorescence;[7,8] third, the size of MNPs can be easily regulated, and thus MNPs accumulate in tumor tissues through enhanced permeability and retention (EPR) effects;[9,10] In light of these characteristics, MNPs have attracted a great deal of attention in biosensing and medical diagnostics.

The partial pressure of oxygen within a living body participates in physiological action and pathological alteration. The partial pressure of oxygen is maintained at physiological levels by homeostatic systems,[11–15] and an imbalance of oxygen homeostasis has been reported to cause various diseases. In particular, hypoxia profoundly relates to cerebrovascular disease,[16,17] cardiovascular disease,[18–20] and solid tumors.[21] Thus, hypoxic microenvironments are of special interest in the study of disease-specific factors.

Emission under hypoxic conditions has been recognized to be a useful characteristic that is applicable to imaging of disease-relevant hypoxia. Recently, we proposed an emission system under hypoxic conditions using indolequinone derivative.[22] **IQ-Cou** consisting of a hypoxia-sensitive indolequinone parent unit,[23–25] two fluorescent coumarin chromophores and a 2,6-bis(hydroxymethyl)-*p*-cresol linker could suppress the fluorescence emission of coumarin moieties by the intramolecular quenching function of indolequinone. Under hypoxic conditions, **IQ-Cou** underwent a one-electron reduction to release fluorescent coumarin chromophores as the action of reduction enzyme, resulting in intense fluorescence emission. Thus, **IQ-Cou** is a promising candidate as a hypoxia-specific fluorescence probe; however, absorption and emission of the coumarin fluorophore in the shorter wavelength region remained to be improved for further biological applications.

The context of previous research prompted us to construct a hypoxia-specific emission system with a longer emission wavelength. We designed and prepared a fluorescein-labeled avidin–biotin system conjugated with indolequinone and MNPs, which are key functional units for regulating fluorescence emission and providing hypoxia-selective emission. The avidin–biotin system can provide multicolor fluorescence probe by means of various avidin molecules bearing a wide variety of differently colored fluorophores.

* Corresponding authors. Tel.: +81 75 383 2500; fax: +81 75 383 2501 (K.T.).
E-mail addresses: tanabeka@scl.kyoto-u.ac.jp (K. Tanabe), nishimot@scl.kyoto-u.ac.jp (S. Nishimoto).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.04.048

We synthesized an indolequinone derivative bearing a biotin moiety (**QB**), which was immobilized on Fe_3O_4 nanoparticles (**QB@MNP**). Fluorescein-labeled avidin (**AF**) was added to this functionalized MNP to form a fluorescein-labeled MNP (**AF-QB@MNP**) through avidin–biotin binding. Fluorescence of **AF-QB@MNP** was suppressed because of the quenching function of indolequinone and the MNP, while the indolequinone unit of **AF-QB@MNP** underwent enzymatic one-electron reduction in a hypoxia-selective manner to liberate the fluorescein-labeled avidin unit so as to give intense fluorescence emission (see Fig. 1).

2. Results and discussion

2.1. Synthesis and identification

The preparation of MNP conjugated with indolequinone and biotin units (**QB@MNP**) is outlined in Scheme 1. Nitroindole derivative **3** was obtained by alkylation of commercially available hydroxyindole **1** and subsequent nitration. After reduction of the nitro group and protection of the hydroxyl group of **3**, the ester group was converted to primary alcohol to give **6**. Oxidation of **6** and condensation with biotin gave indolequinone derivative **8**. Deprotection produced **QB**,[26] which was converted to the activated ester **PNP-QB** by reaction with *p*-nitrophenyl chloroformate. Finally, **PNP-QB** was coupled with MNPs bearing an alkyl amino group[27,28] to give **QB@MNP**.

In order to confirm the immobilization of indolequinone and biotin units (**QB**) on the surface of MNPs, FT-IR measurements were performed. Figure 2 compares the FT-IR spectra of unmodified MNP and **QB@MNP**. The strong band at around 600 cm^{-1} was assigned to the Fe–O stretching vibrations related to the magnetic core,[17] and the band at around 1000 cm^{-1} corresponds to the Si–O stretching vibration.[29,30] It is striking that the bands at 1600–1700 cm^{-1} observed for **QB@NMP** were attributed to the C=O stretching vibration,[31] indicating that **QB** units were immobilized on the surface of MNPs. In addition, the number of **QB** immobilized on an MNP was estimated to be 9.3–14.7 molecules by means of sodium ditionite[32,33] ($Na_2S_2O_4$) treatment of **QB@NMP** and 4-hydoroxyazobenzene-2′-carboxylic acid[34,35] (**HABA**) analysis (Fig. S3). The average diameters of **QB@MNP** were 9.5 ± 2.3 nm as measured by the TEM images (Fig. 2B).

2.2. Fluorescence characteristics

To evaluate the fluorescence characteristics, we measured the fluorescence intensity of fluorescein-labeled avidin (**AF**) in the presence of biotin, **QB@MNP**, or the mixture of biotin and unmodified MNPs. As shown in Figure 3, intense fluorescence emission of **AF** in the presence of biotin was observed, while addition of **QB@MNP** diminished the fluorescence emission. Considering the strong emission of **AF** in the presence of the mixture of biotin and unmodified MNPs, these fluorescence characteristics clearly indicate that complexation of **AF** with **QB@MNP** via avidin–biotin binding occurred to form **AF-QB@MNP**, leading that the fluorescence of **AF** is quenched by the neighboring **QB@MNP**. Thus, it is predicted that fluorescence emission will be restored upon the enzymatic one-electron reduction of **AF-QB@MNP** to release fragment of **AF** with biotin unit.

In the separate experiment, we also characterized fluorescence emission of **AF** in the presence of **QB** to identify the detail of fluorescence quenching of **AF-QB@MNP**. As shown in Figure S1, we confirmed that fluorescence of **AF** was moderately suppressed by the addition of **QB**, which was located close proximity to fluorophore through avidin–biotin binding. Consistent with previous reports,[22] this result strongly indicates that indolequinone unit as well as **MNP** acts as an efficient quencher of fluorescence of **AF**.

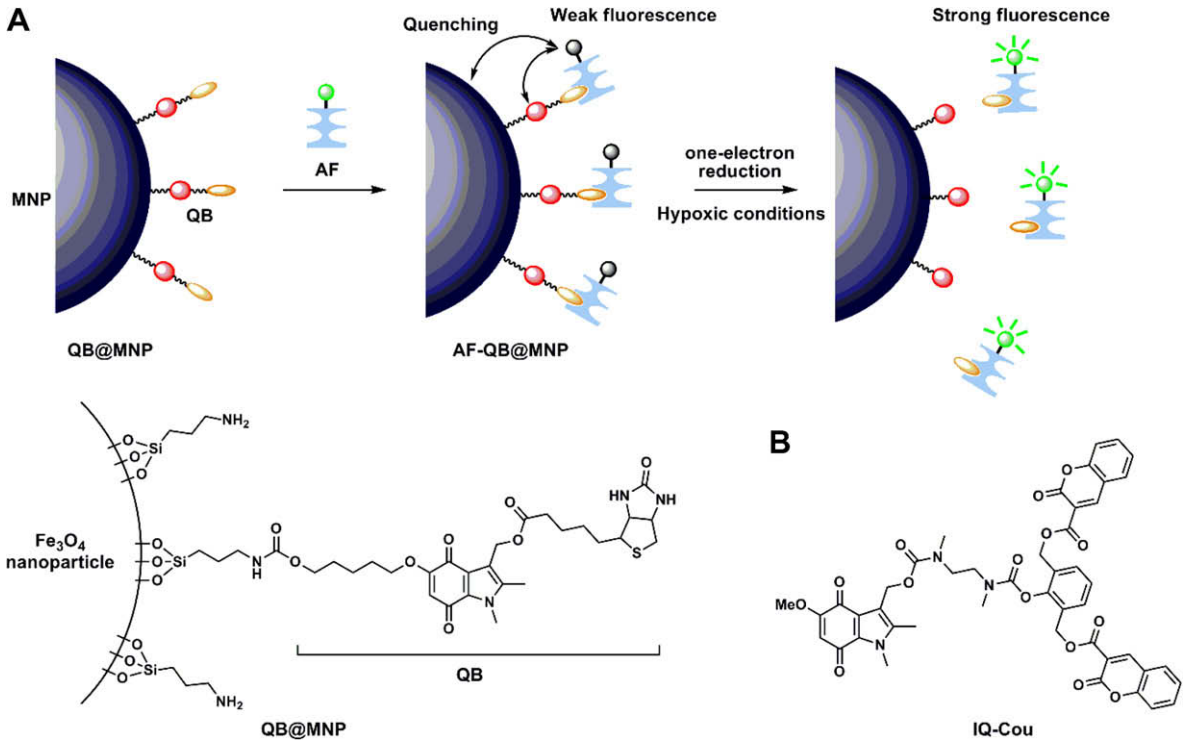
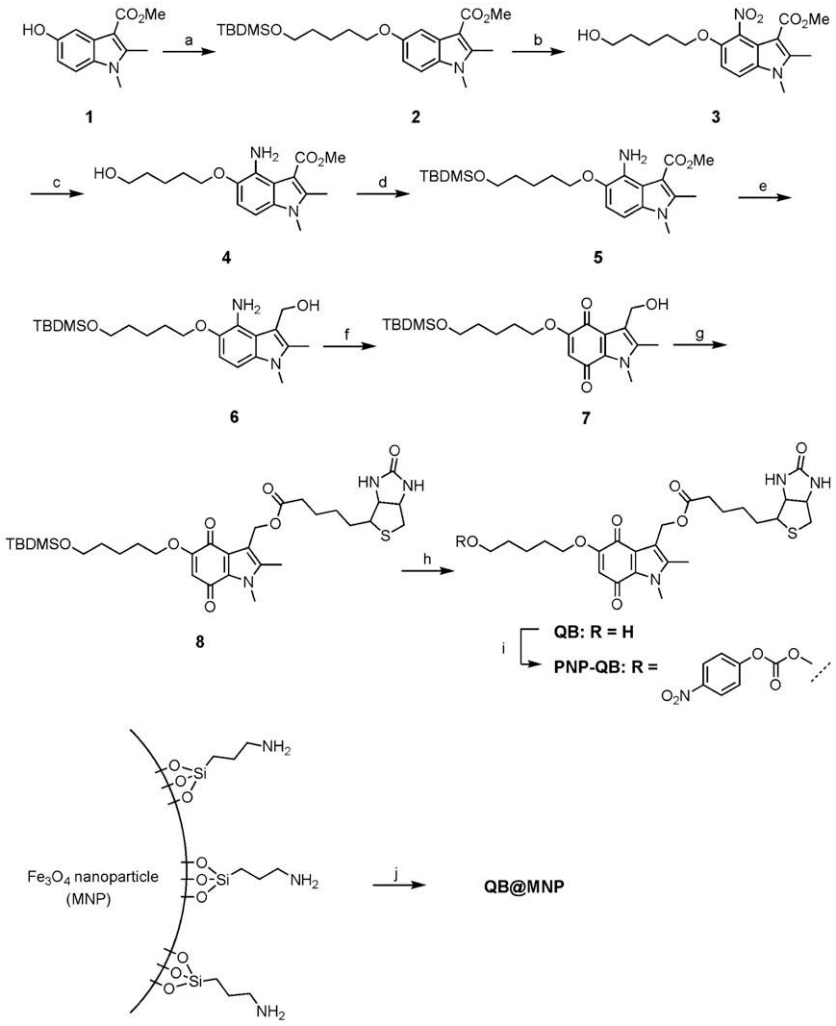


Figure 1. (A) Schematic illustration of hypoxia-specific emission system using an **AF-QB@MNP**. **AF-QB@MNP** consists of fluorescein-labeled avidin (**AF**) and a magnetic nanoparticle (MNP) conjugated with indolequinone–biotin linker (**QB**). The enzymatic one-electron reduction induces activation of **AF-QB@MNP** to enhance the fluorescence in a hypoxia-selective manner. (B) Chemical structure of **IQ-Cou**.



Scheme 1. Reagents and conditions: (a) 5-Bromo-1-*tert*-butyldimethylsilyloxypentane, NaH, DMF, 0 °C to room temperature, overnight, 38%; (b)HNO_3, AcOH, CH_2Cl_2, 0 °C to room temperature, 4 h, 63%; (c)Sn, HCl, EtOH, reflux at 80 °C, 2 h, 95%; (d) *tert*-butyldimethylsilylchloride, imidazole, DMF, 0 °C to room temperature, 3.5 h, 85%; (e) lithium aluminium hydride, THF, 0 °C, 30 min; (f) Fremy's salt, NaH_2PO_4, THF, 0 °C, 20 min, 79% (two steps); (g) biotin, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate, *N*,*N*′-diisopropylethylamine, 4-(dimethylamino)pyridine, DMF, 0 °C to room temperature, overnight, 93%; (h) AcOH–H_2O–THF (13:7:3), room temperature, overnight, 81%; (i) *p*-nitrophenyl chloroformate, triethylamine, CH_2Cl_2, 0 °C to room temperature, 2 h, (33%) (j) **PNP-QB**, DMF, room temperature, 3 days.

2.3. Enzymatic activation

To evaluate the reduction characteristics and fluorescence properties of **AF-QB@MNP** under biological conditions, we conducted an enzymatic reduction. We treated **AF-QB@MNP** with NADPH:cytochrome P450 reductase and cofactor β-NADPH for 5 min at 37 °C under hypoxic or aerobic conditions, and measured the fluorescence spectrum after the quenching of the enzymatic reaction. Figure 4 summarizes the fluorescence intensities at 515 nm, which were normalized with the fluorescence obtained from an untreated sample. An enhanced fluorescence emission was observed for the sample incubated under hypoxic conditions. This result strongly suggests that fragmentation occurred at the indolequinone linker during enzymatic one-electron reduction, resulting in separation of quencher unit of indolequinone-modified MNP and fluorophore of **AF** to restore the fluorescence of **AF**. In contrast, the extent of enhanced fluorescence intensity diminished significantly upon aerobic treatment. According to the previous results,[22] molecular oxygen scavenges semiquinone radicals,[36] which was formed by enzymatic one-electron reduction of the indolequinone derivative, to inhibit the reductive fragmentation. Thus, **AF-QB@MNP** was activated by NADPH:cytochrome P450 reductase to restore its fluorescence emission in a hypoxia-selective manner.

3. Conclusion

In summary, we have designed and prepared a new class of functional magnetic nanoparticles (MNP) linked to fluorophore-labeled avidin through hypoxia-responsive indolequinone–biotin derivative (**AF-QB@MNP**). The fluorescence of fluorescein-labeled avidin (**AF**) was dramatically decreased by the addition of Fe_3O_4 nanoparticles modified with an indolequinone derivative bearing biotin unit **(QB@MNP)**. This result strongly indicates that complexation between **AF** and QB-modified MNPs via avidin–biotin binding led to efficient quenching of fluorescence. **AF-QB@MNP** showed significant enhancement of emission upon enzymatic reduction under hypoxic conditions, whereas an enhancement in fluorescence emission was suppressed upon aerobic treatment. In view of these fluorescence properties, **AF-QB@MNP** is a promising candidate as a fluorescence probe of reduction environment and disease-relevant hypoxia. Our current study focuses on identification of activation mechanism by laser flash photolysis and pulse radiolysis to establish guides for molecular design of indolequinone derivatives with high

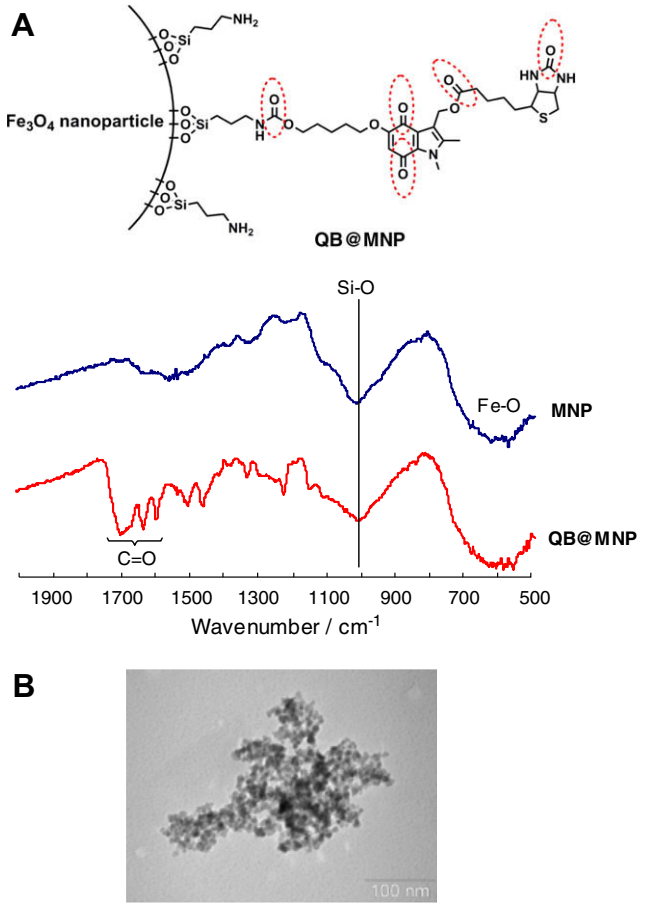


Figure 2. (A) Comparison of FT-IR spectra of unmodified Fe_3O_4 nanoparticle (blue line) and **QB@MNP** (red line). (B) TEM images of **QB@MNP** (Avg. 9.5 nm, S.D. 2.3 nm).

performance, and applications of **AF-QB@MNP** and its related probes to *in vivo* optical imaging of hypoxia.

4. Experimental

4.1. General methods

All reactions were carried out under a dry nitrogen atmosphere using freshly distilled solvents unless otherwise noted. Reagents were purchased from Aldrich, Wako pure chemical industries and Nacalai tesque, and used as received. NADPH:cytochrome P450 reductase and β-NADPH coenzyme were obtained from Oxford Biomedical Research and Oriental Yeast Co., respectively. Ultrapure water was obtained from YAMATO WR-600A. Precoated TLC (Merck silica gel 60 F_{254}) plates were used for monitoring the reactions. Column chromatography was carried out on Wakogel C-300 (Wako pure chemical industries). ^{1}H NMR spectra were measured with JEOL JMN-AL-300 (300 MHz) or JEOL JMN-AL-400 (400 MHz) spectrophotometers at ambient temperature. ^{13}C NMR spectra were measured with JEOL JMN-AL-300 (75.5 MHz) or JEOL JMN-AL-400 (100 MHz) spectrophotometers at ambient temperature. Coupling constants (J values) are reported in hertz. The chemical shifts are expressed in ppm downfield from tetramethylsilane, using residual chloroform (δ = 7.24 in ^{1}H NMR, δ = 77.0 in ^{13}C NMR) and dimethylsulfoxide (δ = 2.49 in ^{1}H NMR, δ = 39.5 in ^{13}C NMR) as an internal standard. Multiplicity is designed as singlet (s), doublet (d), triplet (t), doublet–doublet (dd), or multiplet (m). FAB Mass spectra were recorded on JEOL JMS-SX102A spectrometer, using NBA matrix. Fluorescent spectra were recorded on Shimadzu RF-5300PC spectrofluorophotometer at ambient temperature. UV spectra were recorded with a Jasco V-530 UV/vis spectrometer at ambient temperature. FT-IR spectra were recorded on a Shimadzu IR Prestage-21 infrared spectrophotometer using a KBr disk dispersed with the powdered sample. Transmission electron microscopy (TEM) measurements were performed using a JOEL JEM-100SX operated at 100 kV electron beam accelerating voltage. One drop of the sample solution was deposited onto a copper grid and the excess of the droplet was blotted off the grids with filter paper; then the sample was dried under ambient conditions.

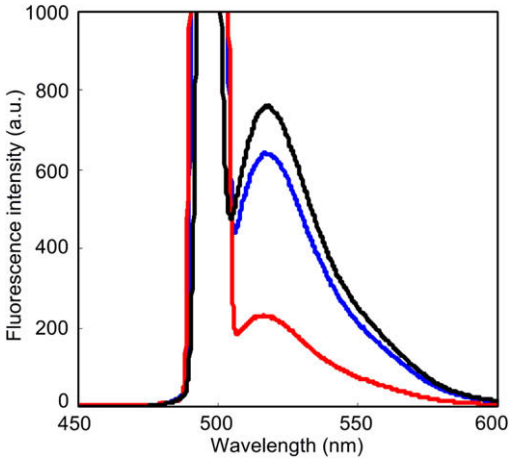


Figure 3. Fluorescence spectra of 500 ng/mL **AF** in the presence of 72.2 nM biotin (black line), **QB@MNP** (263 μg/mL, red line), or the mixture of 72.2 nM biotin and unmodified MNP (222 μg/mL, blue line) in 25 mM phosphate buffer (pH 7.4).

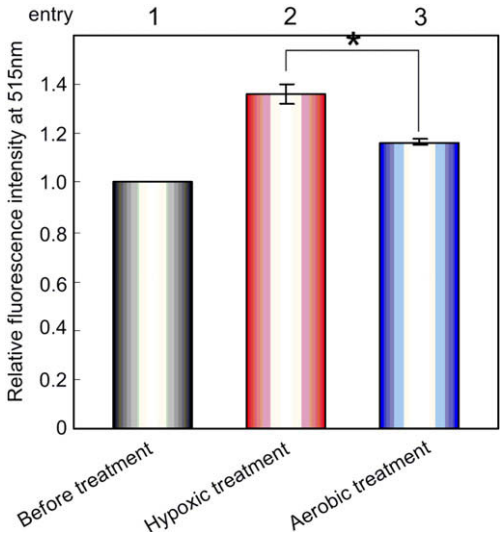


Figure 4. Relative fluorescence intensity normalized with the fluorescence intensity at 515 nm of **AF-QB@MNP** (entry 1). **AF** in the presence of **QB@MNP** in 25 mM phosphate buffer (pH 7.4) was treated by NADPH:cytochrome P450 reductase (22.7 μg/mL) and β-NADPH coenzyme (2 mM) at 37 °C for 5 min under hypoxic (entry 2) and aerobic (entry 3) conditions. These fluorescence intensities were measured with excitation at 495 nm after the reaction. Results are shown with the mean ± S.D. (n = 3) ($^*P$ < 0.01).

4.2. Synthesis

4.2.1. 5-[5-(*tert*-Butyldimethylsilanyloxy)pentoxy]-1,2-dimethyl-1*H*-indole-3-carboxylic acid methyl ester (2)

To a solution of the alcohol **1** (14.5 g, 66 mmol) in DMF (200 mL) cooled in ice-water bath (0 °C) was added 50–72% sodium hydride in oil (2.71 g, 55–79 mmol) followed by stirring at same temperature. To the resultant mixture was added 5-bromo-1-*tert*-butyl-

dimethylsilyloxypentane (31.2 g, 110 mmol) followed by stirring at room temperature for overnight. The reaction was quenched with saturated aqueous $NaHCO_3$ and extracted with EtOAc. The combined organic layer was washed with brine, dried over anhydrous $MgSO_4$, filtered, concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, EtOAc–hexane = 1:10) to give the title compound (10.6 g, 38% yield). A yellow oil; R_f = 0.74 (50% EtOAc–hexane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55 (d, J = 2.4 Hz, 1H), 7.10 (d, J = 8.8 Hz, 1H), 6.80 (dd, J = 2.4, 8.9 Hz, 1H), 3.99 (t, J = 6.6 Hz, 2H), 3.86 (s, 3H), 3.60 (s, 3H), 3.59 (t, J = 6.6 Hz, 2H), 2.67 (s, 3H), 1.78 (m, 2H), 1.50 (m, 4H), 0.84 (s, 9H), 0.00 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.6, 155.1, 145.3, 131.6, 127.3, 112.1, 109.6, 104.7, 103.4, 68.6, 63.1, 50.6, 32.6, 29.7, 29.2, 26.0, 22.4, 18.4, 12.0, −5.3; FABMS m/z 419 [M^+]; HRMS calcd for $C_{23}H_{38}NO_4Si$ [$(M+H)^+$] 420.2565, found 420.2580.

4.2.2. 5-(5-Hydroxypentoxy)-1,2-dimethyl-4-nitro-1*H*-indole-3-carboxylic acid methyl ester (3)

To a solution of the indole **2** (4.33 g, 10 mmol) and acetic acid (1.8 mL, 30 mmol) in CH_2Cl_2 (50 mL) cooled in ice-water bath (0 °C) was added 61% nitric acid (1.2 mL, 15 mmol) followed by stirring at room temperature for 4 h. The reaction was quenched with saturated aqueous $NaHCO_3$ and extracted with $CHCl_3$. The combined organic layer was washed brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, EtOAc–hexane = 4:1) to give the title compound (2.22 g, 63% yield). A brown solid; mp 83–87 °C; R_f = 0.09 (50% EtOAc–hexane); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.73 (d, J = 9.0 Hz, 1H), 7.20 (d, J = 9.0 Hz, 1H), 4.35 (t, J = 5.0 Hz, 1H), 4.09 (t, J = 6.4 Hz, 2H), 3.76 (s, 3H), 3.63 (s, 3H), 3.38 (q, J = 6.1 Hz, 2H), 2.66 (s, 3H), 1.66 (m, 2H), 1.44–1.38 (4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.0, 147.7, 145.5, 132.2, 116.7, 113.3, 109.5, 101.3, 101.1, 70.2, 60.6, 50.0, 32.0, 30.2, 28.5, 21.8, 11.7; FABMS m/z 350 [M^+]; HRMS calcd for $C_{17}H_{23}N_2O_6$ [$(M+H)^+$] 351.1551, found 351.1554.

4.2.3. 4-Amino-5-(5-hydroxypentoxy)-1,2-dimethyl-1*H*-indole-3-carboxylic acid methyl ester (4)

To a solution of **3** (2.21 g, 6.3 mmol) in EtOH (50 mL) were added Sn (3.75 g, 32 mmol) and 35% HCl (13 mL, 147 mmol). The reaction was refluxed with stirring at 80 °C for 2 h. The reaction was quenched with saturated aqueous $NaHCO_3$ and extracted with EtOAc. The combined organic layer was washed with brine, dried over anhydrous $MgSO_4$, filtered, concentrated under reduced pressure to give the title compound (1.91 g, 95% yield). An off-white solid; mp 103–105 °C; R_f = 0.50 (80% EtOAc–hexane); ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.83 (d, J = 8.8 Hz, 1H), 6.59 (d, J = 8.6 Hz, 1H), 5.72 (bs, 2H), 4.35 (t, J = 5.2 Hz, 1H), 3.89 (t, J = 6.5 Hz, 2H), 3.80 (s, 3H), 3.58 (s, 3H), 3.41 (m, 2H), 2.60 (s, 3H), 1.72 (m, 2H), 1.48–1.45 (4H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 166.7, 144.1, 139.2, 133.6, 131.0, 113.5, 111.0, 102.9, 96.6, 69.8, 60.6, 51.0, 32.3, 29.8, 29.1, 22.2, 12.7; FABMS m/z 320 [M^+]; HRMS calcd for $C_{17}H_{25}N_2O_4$ [$(M+H)^+$] 321.1809, found 321.1804.

4.2.4. 4-Amino-5-[5-(*tert*-butyldimethylsilanyloxy)pentoxy]-1,2-dimethyl-1*H*-indole-3-carboxylic acid methyl ester (5)

To a solution of the alcohol **4** (4.0 g, 12.5 mmol) and imidazole (1.84 g, 27 mmol) in DMF (50 mL) cooled in ice-water bath (0 °C) was added *tert*-butyldimethylsilylchloride (3.94 g, 26 mmol) followed by stirring at room temperature for 3.5 h. The reaction was quenched with saturated aqueous $NaHCO_3$ and extracted with EtOAc. The combined organic layer was washed with brine, dried over anhydrous $MgSO_4$, filtered, concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, EtOAc–hexane = 1:4) to give the title compound (4.61 g, 85% yield). An off-white solid; mp 90–91 °C; R_f = 0.91 (80% EtOAc–hexane); ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.80 (d, J = 8.6 Hz, 1H), 6.56 (d, J = 8.6 Hz, 1H), 5.68 (bs, 2H), 3.86 (m, 2H), 3.58 (s, 3H), 3.56 (m, 5H) 3.28 (s, 3H), 2.57 (s, 3H), 1.69 (m, 2H), 1.46 (m, 4H), 0.83 (s, 9H), 0.01 (s, 6H); ^{13}C NMR (75.5 MHz, DMSO-d_6), δ 166.7, 144.0, 139.2, 133.6, 131.0, 113.5, 111.0, 102.9, 96.6, 69.7, 62.4, 51.0, 32.1, 29.8, 29.0, 25.8, 22.0, 17.9, 12.7, -5.3; FABMS m/z 435 [$(M+H)^+$]; HRMS calcd for $C_{23}H_{39}N_2O_4Si$ [$(M+H)^+$] 435.2674, found 435.2673.

4.2.5. {4-Amino-5-[5-(*tert*-butyldimethylsilanyloxy)pentoxy]-1,2-dimethyl-1*H*-indole-3-yl}methanol (6)

To a solution of the ester **5** (4.61 g, 11 mmol) in THF (50 mL) cooled in ice-water bath (0 °C) was added lithium aluminium hydride (800 mg, 21 mmol) followed by stirring at the same temperature for 30 min. The reaction was quenched by ice-water. The reaction mixture was extracted with EtOAc. The combined organic layer was washed with brine, died over anhydrous $MgSO_4$, filtered, concentrated under reduced pressure. The given compound was immediately reacted for the next step without purification.

4.2.6. 5-[5-(*tert*-Butyldimethylsilanyloxy)pentoxy]-3-hydroxymethyl-1,2-dimethyl-1*H*-indole-4,7- dione (7)

To a solution of the 4-aminoindole **6** (4.31 g, crude) and trioctylmethylammonium chloride (8.6 g, 21 mmol) in THF (22 mL) cooled in ice-water bath (0 °C) was added potassium nitrosodisulfonate (Fremy's salt) (7.18 g, 27 mmol) in aqueous sodium dihydrogenphosphate (0.3 M, 22 mL) followed by stirring at the same temperature for 20 min. The reaction was quenched saturated aqueous $NaHCO_3$, extracted with EtOAc. The combined organic layer was washed with brine, dried over anhydrous $MgSO_4$, filtered, concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, EtOAc–hexane = 1:2) to give the title compound (3.52 g, 79% yield in two steps). A red oil; R_f = 0.63 (80% EtOAc–hexane); ^{1}H NMR (400 MHz, DMSO-d_6), δ 5.68 (s, 1H), 4.58 (d, J = 4.9 Hz, 1H), 4.54 (s, 2H), 3.88 (t, J = 6.3 Hz, 2H), 3.80 (s, 3H), 3.56 (t, J = 6.0 Hz, 2H), 2.20 (s, 3H), 1.74–1.68 (2H), 1.48–1.39 (4H), 0.83 (s, 9H), 0.00 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.5, 159.0, 154.7, 135.0, 126.0, 122.5, 120.4, 104.5, 69.4, 62.3, 54.4, 31.9, 31.8, 27.6, 25.8, 21.8, 17.9, 9.2, -5.3; FABMS m/z 421 [M^+]; HRMS calcd for $C_{22}H_{36}NO_5Si$ [$(M+H)^+$] 422.2357, found 422.2354.

4.2.7. Indolequinone derivatives conjugated with biotin (8)

To a solution of the indolequinone **7** (1.73 g, 4.1 mmol) and biotin (2.03 g, 8.3 mmol) in DMF (23 mL) cooled in ice-water bath (0 °C) were added *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (2.71 g, 8.4 mmol), 4-(dimethylamino)pyridine (612 mg, 5 mmol), and *N*,*N*′-diisopropylethylamine (1.44 mL, 8.2 mmol) followed by stirring at room temperature for overnight. The reaction was quenched with saturated aqueous $NaHCO_3$ and extracted with $CHCl_3$. The combined organic layer was washed with brine, dried over anhydrous $MgSO_4$, filtered, concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, MeOH–$CHCl_3$ = 1:20) to give the title compound (2.48 g, 93% yield). An orange solid, mp 109–110 °C; R_f = 0.54 (9% MeOH–$CHCl_3$); ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.37 (s, 1H), 6.32 (s, 1H), 5.72 (s, 1H), 5.10 (s, 2H), 4.26 (t, J = 7.6 Hz, 1H), 4.08 (m, 1H), 3.89 (t, J = 6.3 Hz, 2H), 3.81 (s, 3H), 3.81 (m, 2H), 3.02 (m, 1H), 2.67 (dd, J = 12.4, 5.1 Hz, 1H), 2.53 (d, J = 12.4 Hz, 1H), 2.24–2.21 (5H), 1.72–1.69 (2H), 1.49–1.41 (8H), 1.27 (m, 2H), 0.82 (s, 9H), 0.00 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.3, 177.1, 172.7, 162.6, 158.5, 138.4, 128.1, 120.8, 114.8, 106.9, 69.1, 62.3, 61.0, 59.1, 56.0, 55.3, 40.1, 33.3, 32.1, 31.9, 27.91, 27.89, 27.6, 25.8, 24.6, 21.8, 17.9,

9.2, −5.3; FABMS *m/z* 648 [(M+H)$^+$]; HRMS calcd for $C_{32}H_{50}N_3O_7SSi$ [(M+H)$^+$] 648.3133, found 648.3135.

4.2.8. Preparation of QB

To a solution of the indolequinone derivative **8** (209 mg, 0.32 mmol) was added a solution of 10 mL AcOH–H_2O–THF (13:7:3) followed by stirring at room temperature for overnight. The reaction was quenched with saturated aqueous $NaHCO_3$ and extracted with $CHCl_3$. The combined organic layer was washed with brine, dried over anhydrous $MgSO_4$, filtered, concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, MeOH–$CHCl_3$ = 10:1) to give the title compound (140 mg, 81% yield). An orange solid; mp 163–166 °C; R_f = 0.23 (9% MeOH–$CHCl_3$); ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.38 (s, 1H), 6.33 (s, 1H), 5.73 (s, 1H), 5.12 (s, 2H), 4.37 (t, *J* = 4.9 Hz, 1H), 4.27 (t, *J* = 7.8 Hz, 1H), 4.09 (m, 1H), 3.90 (t, *J* = 6.3 Hz, 2H), 3.83 (s, 3H), 3.39 (m, 2H), 3.04 (m, 1H), 2.79 (dd, *J* = 12.4, 5.1 Hz, 1H), 2.55 (d, *J* = 12.4 Hz, 1H), 2.26–2.23 (5H), 1.73–1.69 (2H), 1.54–1.39 (8H), 1.29–1.28 (2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.3, 177.2, 172.7, 162.6, 158.4, 138.5, 128.1, 120.8, 114.8, 106.9, 69.2, 61.0, 60.5, 59.1, 56.0, 55.3, 40.1, 33.2, 32.1, 32.0, 27.91, 27.89, 27.8, 24.5, 22.0, 9.1; FABMS *m/z* 534 [(M+H)$^+$]; HRMS calcd for $C_{26}H_{36}N_3O_7S$ [(M+H)$^+$] 534.2268, found 534.2286.

4.2.9. Preparation of PNP-QB

To a solution of the **QB** (93 mg, 0.174 mmol) in CH_2Cl_2 (5 mL) cooled in ice-water bath (0 °C) were added *p*-nitrophenyl chloroformate (PNP–Cl, 211 mg, 1.047 mmol) and triethylamine (TEA, 144 μL, 1.035 mmol) followed by stirring at 0 °C to room temperature for 2 h. After the reaction, the resultant mixture was evaporated to remove the solvent. The residue was purified by flash column chromatography (silica gel, MeOH–$CHCl_3$ = 1:20) to give the title compound (40 mg, 33% yield). An orange oil; R_f = 0.31 (9% MeOH-$CHCl_3$); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.29 (d, *J* = 9.3 Hz, 2H), 7.55 (d, 9.3 Hz, 2H), 6.38 (s, 1H), 6.33 (s, 1H), 5.75 (s, 1H), 5.12 (s, 2H), 4.27 (t, *J* = 6.3 Hz, total 3H (2H overlapped with 1H)), 4.09 (t, *J* = 5.1 Hz, 1H), 3.94 (t, *J* = 6.3 Hz, 2H), 3.83 (s, 3H), 3.04 (m, 1H), 2.79 (dd, *J* = 12.4 Hz, 4.9 Hz, 1H), 2.55 (d, *J* = 12.4 Hz, 1H), 2.23 (m, 5H), 1.78–1.73 (4H), 1.52–1.27 (8H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.3, 177.1, 172.7, 162.6, 158.4, 155.3, 152.0, 145.1, 138.5, 128.1, 125.3, 122.6, 120.8, 114.8, 107.0, 79.1, 69.0, 61.0, 59.1, 56.0, 55.3, 40.1, 33.2, 32.1, 27.90, 27.87, 27.5, 27.4, 24.5, 21.7, 9.1; FABMS *m/z* 699 [(M+H)$^+$]; HRMS calcd for $C_{33}H_{39}N_4O_{11}S$ [(M+H)$^+$] 699.2331, found 699.2335.

4.2.10. Preparation of iron oxide magnetic nanoparticles bearing alkyl amino group (MNP)

Fe_3O_4 magnetic nanoparticles bearing alkylamino group were prepared as reported.[27,28]

4.2.11. Preparation of QB@MNP

To a precipitate of MNP (32 mg) was added **PNP-QB** (16 mg, 0.023 mmol) in DMF (16 mL) followed by stirring at room temperature for 3 days. After the reaction, the resultant mixture was centrifuged (6200 rpm, 30 min) to remove the solvent. Subsequently, the residue was washed by DMF and re-centrifuged (6200 rpm, 30 min) to remove the solvent. This washing treatment was repeated as seven times. After that, the residual MNP sample was dispersed in DMF (46.5 ± 1.7 mg/mL), and reserved or used for the experimental protocols.

4.3. HABA analysis for quantitative determination of the number of biotin immobilized on MNP

To a solution of 500 μL **QB@MNP** (46.5 ± 1.7 mg/mL in DMF) was added 300 μL $Na_2S_2O_4$ solution (10 mM in 25 mM phosphate buffer pH 11.8) followed by vortex, adding PBS (200 μL) to quench the reaction, and centrifugation (6200 rpm, 30 min) to remove and gather the solvent, which includes a released biotin. This protocol was repeated twice. The gathered solvent was scaled up to 5 mL with PBS. As a control sample, a treatment solution processed MNP with $Na_2S_2O_4$ was prepared in a similar manner. To a solution of avidin (final concentration; 10 μM) including HABA (final concentration; 1.5 mM) was added the treatment solution processed **QB@MNP** or MNP with $Na_2S_2O_4$ (20 μL picked up from 5 mL solution, respectively). The total volume of these mixtures was scaled up and integrated to 100 μL (resulting that the treatment solution was diluted by 20%). Subsequently, the absorbance at 500 nm of these mixtures was monitored by UV/vis spectrometer.

A calibration curve was determined by the monitoring the change of the absorbance at 500 nm in adding biotin (10, 20, 30, 40 μM) to a solution of avidin (final concentration; 10 μM) including HABA (final concentration; 1.5 mM). Amount of released biotin was quantified by using the linear fit ($y = 0.205x + 2.22$, $R^2 = 0.986$; *y*: the absorbance at 500 nm, *x*: the concentration of biotin).

4.4. Fluorescence measurements

Fluorescence spectra of 500 ng/mL **AF** in the presence of 72.2 nM biotin, **QB@MNP** (5 μL of 46.5 ± 1.7 mg/mL), and the mixture of 72.2 nM biotin and unmodified MNP (5 mL of 44.4 ± 2.5 mg/mL) in 25 mM phosphate buffer (pH 7.4) were taken on a SHIMADZU RF-5300PC fluorescence spectrophotometry with excitation at 495 nm.

4.5. Bioreductive activation of AF-QB@MNP

A mixture of β-NADPH, **AF** and **QB@MNP** (final concentration: 2 mM, 500 ng/mL and 465 ± 17 μg/mL, respectively) in 25 mM phosphate buffer (pH 7.4), which was preliminarily purged with argon for 30 min, were kept in anaerobic environmental glove box for 2 h at ambient temperature to establish hypoxia. To the resulting solution was added NADPH:cytochrome P450 reductase (final concentration: 22.7 μg/mL) and incubated for 5 min at 37 °C. After the reaction, aliquots were sampled and diluted 10% by 25 mM phosphate buffer (pH 7.4) for quenching the reaction and measuring the fluorescent spectra. Fluorescence spectra were taken with excitation at 495 nm and detected at 515 nm. As a control sample, air-saturated buffer was used and analyzed in a similar manner. All fluorescence intensities were normalized with intensity at 515 nm of **AF** (final concentration, 500 ng/mL) in the presence of **QB@MNP** (final concentration; 465 ± 17 μg/mL).

Acknowledgments

This study is a part of joint researches, which are focusing on development of basis of technology for establishing COE of nanomedicine, carried out through Kyoto City Collaboration of Regional Entities for Advancement of Technology Excellence (CREATE) assigned by Japan Science and Technology Agency (JST).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.04.048.

References and notes

1. Polito, L.; Colombo, M.; Monti, D.; Melato, S.; Caneva, E.; Prosperi, D. *J. Am. Chem. Soc.* **2008**, *130*, 12712.
2. Huh, Y.-M.; Lee, E.-S.; Lee, J.-H.; Jun, Y.-W.; Kim, P.-H.; Yun, C.-O.; Kim, J. 0-H.; Suh, J.-S.; Cheon, J. *Adv. Mater.* **2007**, *19*, 3109.

3. Weissleder, R.; Kelly, K.; Sun, E. Y.; Shtatland, T.; Josephson, L. *Nat. Biotechnol.* **2005**, *23*, 1418.
4. Vehseh, O.; Sun, C.; Gunn, J.; Kohler, N.; Gabikan, P.; Lee, D.; Narayan, B.; Ellenbogen, R.; Sze, R.; Hllahan, A.; Olson, J.; Zhang, M. *Nano Lett.* **2005**, *5*, 1003.
5. Zhao, M.; Kircher, M. F.; Josephson, L.; Weissleder, R. *Bioconjugate Chem.* **2002**, *13*, 840.
6. Shen, X.-C.; Fang, X.-Z.; Zhou, Y.-H.; Liang, H. *Chem. Lett.* **2004**, *33*, 1468.
7. Li, L.; Choo, E. S. G.; Liu, Z.; Ding, J.; Xue, J. *Chem. Phys. Lett.* **2008**, *461*, 114.
8. Mandal, S. K.; Lequeux, N.; Rotenberg, B.; Tramier, M.; Fattaccioli, J.; Bibette, J.; Dubertret, B. *Langmuir* **2005**, *21*, 4175.
9. Matsumura, Y.; Maeda, H. *Cancer Res.* **1986**, *46*, 6387.
10. Iyer, A. K.; Khaled, G.; Fang, J.; Maeda, H. *Drug Discovery Today* **2006**, *11*, 812.
11. Semenza, G. L. *Annu. Rev. Cell. Dev. Biol.* **1999**, *15*, 551.
12. Ke, Q.; Costa, M. *Mol. Pharmacol.* **2006**, *5*, 1469.
13. Li, X. Y.; Takasaki, C.; Satoh, Y.; Kimura, S.; Yasumoto, K.; Sogawa, K. *J. Biochem.* **2008**, *144*, 555.
14. Downie, B. R.; Sanchez, A.; Knotgen, H.; Contreras-Jurado, C.; Gymnopoulos, M.; Weber, C.; Stuhmer, W.; Pardo, L. A. *J. Biol. Chem.* **2008**, *283*, 36234.
15. Masson, N.; Ratcliffe, P. J. *J. Cell Sci.* **2003**, *116*, 3041.
16. Manabe, T.; Katayama, T.; Sato, N.; Gomi, F.; Hitomi, J.; Yanagita, T.; Kudo, T.; Honda, A.; Mori, Y.; Matsuzaki, S.; Imaizumi, K.; Meyeda, A.; Tohyama, M. *Cell Death Differ.* **2003**, *10*, 698.
17. Sato, N.; Hori, O.; Yamaguchi, A.; Lambert, J.-C.; Chartier-Harlin, M.-C.; Robinson, P. A.; Delacourte, A.; Schmidt, A. M.; Furuyama, T.; Imaizumi, K.; Tohyama, M.; Takagi, T. *J. Neurochem.* **1999**, *72*, 2498.
18. Haunstetter, A.; Izumo, S. *Circ. Res.* **1998**, *82*, 1111.
19. Chen, C.-J.; Yu, W.; Fu, Y.-C.; Wang, X.; Li, J.-L.; Wang, W. *Biochem. Biophys. Res. Commun.* **2009**, *378*, 389.
20. Bonavita, F.; Stefanelli, C.; Giordano, E.; Columbaro, M.; Facchini, A.; Bonafe, F.; Caldarera, C. M.; Guarnieri, C. *FEBS Lett.* **2003**, *536*, 85.
21. Vaupel, P.; Kallinowski, F.; Okunieff, P. *Cancer Res.* **1989**, *49*, 6449.
22. Tanabe, K.; Hirata, N.; Harada, H.; Hiraoka, M.; Nishimoto, S.-I. *ChemBioChem.* **2008**, *9*, 426.
23. Everett, S. A.; Swann, E.; Naylor, M. A.; Stratford, M. R. L.; Patel, K. B.; Tian, N.; Newman, R. G.; Vojnovic, B.; Moody, C. J.; Wardman, P. *Biochem. Pharmacol.* **2002**, *63*, 1629.
24. Newsome, J. J.; Swann, E.; Hassani, M.; Bray, K. C.; Slawin, A. M. Z.; Beall, H. D.; Moody, C. J. *Org. Biomol. Chem.* **2007**, *5*, 1629.
25. Tanabe, K.; Makimura, Y.; Tachi, Y.; Imagawa-Sato, A.; Nishimoto, S. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2321.
26. Functions of indolequinone and biotin were maintained even in **QB**. The fluorescence of **AF** in the presence of **QB** was suppressed because of quenching function of indolequinone. In contrast, fluorescence of **AF** was restored by treatment of NADPH:cytochrome P450 reductase under hypoxic conditions (see Figs. S1 and S2).
27. Naka, K.; Narita, A.; Tanaka, H.; Chujo, Y.; Morita, M.; Inubushi, T.; Nishimura, I.; Hiruta, J.; Shibayama, H.; Koga, M.; Ishibashi, S.; Seki, J.; Kondoh-Kizaka, S.; Hiraoka, M. *Polym. Adv. Technol.* **2008**, *19*, 1421.
28. Narita, A.; Naka, K.; Chujo, Y. *Colloids Surf., A* **2009**, *336*, 46.
29. Bruce, I. L.; Sen, T. *Langmuir* **2005**, *21*, 7029.
30. Ma, M.; Zhang, Y.; Yu, W.; Shen, H.; Zhang, H.; Gu, N. *Colloids Surf., A* **2003**, *212*, 219.
31. Itoh, S.; Takada, N.; Ando, T.; Haranou, S.; Huang, X.; Uenoyama, Y.; Ohshiro, Y.; Komatsu, M.; Fukuzumi, S. *J. Org. Chem.* **1997**, *62*, 5898.
32. Hernick, M.; Borch, R. F. *J. Med. Chem.* **2003**, *46*, 148.
33. Hernick, M.; Flader, C.; Borch, R. F. *J. Med. Chem.* **2002**, *45*, 3540.
34. Green, N. M. *Biochem. J.* **1965**, *94*, 23c.
35. Morpurgo, M.; Hofstetter, H.; Bayer, E. A.; Wilchek, M. *J. Am. Chem. Soc.* **1998**, *120*, 12734.
36. We monitored the enzymatic reduction of **QB** under aerobic conditions, and confirmed that there was not remarkable degradation of indolequinone chromophore (see Fig. S4).