Electronic Supplementary Information (ESI)

Biofunctional TiO_2 Nanoparticle Mediated Photokilling of Cancer Cells Using UV Irradiation

Kazusa Matsui[1], Miki Karasaki[1], Maiko Segawa[1], Hwang Sang Youn[2], Tsutomu Tanaka[2], Chiaki Ogino[1*] and Akihiko Kondo[1]

[1]Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodaicho, Nada, Kobe 657-8501, Japan

[2]Organization of Advanced Science and Technology, Kobe University, 1-1 Rokkodaicho, Nada, Kobe 657-8501, Japan

Corresponding author: Chiaki Ogino

Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodaicho, Nada, Kobe 657-8501, Japan

Tel/Fax: +81-78-803-6193

E-mail: ochiaki@port.kobe-u.ac.jp

Construction of Expression Plasmid

KOD plus DNA polymerase (Toyobo) was used for PCR, and all PCR-amplified sequences were verified by DNA sequencing. The expression plasmid for anti-epidermal growth factor receptor (EGFR) antibody was constructed as follows: the gene encoding aEGFR VHH was obtained by PCR from genomic DNA of pUC-la1 vector (Shishido, T.; Azumi, Y.; Nakanishi, T.; Umetsu, M.; Tanaka, T.; Ogino, C.; Fukuda, H.; Kondo, A.; *J. Biochem*. 2009, 46, 867-874) using the 5' primer (5'-AACCATGGCCCAGGTGCAGCTGCAGGAAAGCGGC-3') and 3' primer (5'-CTGAATTCCTAACCGCCAGTTTCCGGCAGAGAACCGCCATGGTGGTGATG ATGGTGCGAG-3'), and then subcloned into the *Nco*I/*Eco*RI sites of pET32b(+) (Novagen) to yield pET32b-la.

Expression and purification of anti-EGFR VHH

Plasmid pET32b-la was transformed into *E. coli* BL21 (DE3) (Novagen). Cells were grown in LB medium to an OD (600 nm) value of 0.8, at which time expression of the protein was induced by the addition of isopropyl-b-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. After growth for an additional 16 h at 27°C, the cells were harvested by centrifugation (15000 rpm, 4°C, 30 min). The cell pellets were resuspended in 50 mM $NaPO_4$ containing 300 mM NaCl (pH 7.0) and lysed by sonication. Anti-EGFR VHH was purified from the soluble faction of the lysate by TARON metal affinity resins (Clontech) according to the manufacturer's protocol, and

dialyzed against 20 mM Tris-HCl containing 150 mM NaCl (pH 8.0). The concentration of purified anti-EGFR VHH was determined using a BCA protein assay kit (Pierce).

Preparation of polyacrylic acid (PAA)-modified TiO_2 nanoparticles

Two kinds of acidic TiO_2 solution were purchased from Ishihara Sangyo Kaisha, Ltd. (Japan). The diameters of TiO_2 (STS-100) and TiO_2 (STS-01) are 10 nm 30 nm, respectively. A 1-ml aliquot of TiO_2 solution was mixed with 37.5 ml N,N-dimethylformamide (DMF), followed by 4 ml DMF containing 100 mg/ml PAA (average molecular weight: 5000, Wako Pure Chemical Industries, Japan) and incubated at 150°C for 5 h. After cooling the mixture to room temperature, twice volume of 99.5% acetone was added and the mixture was incubated at room temperature for 1 h to separate reacted TiO_2 nanoparticles. The precipitate was recovered by centrifugation (4,000 rpm, 20 min). Then 99.5% ethanol was added to supernatant for precipitation of PAA-modified TiO_2 and then centrifuged again. The precipitants were solubilized in 20 mM HEPES buffer (pH 7.4).

Construction of anti-EGFR antibody-modified TiO_2 nanoparticles

The PAA-TiO_2 suspension (6 w/v%, 2 ml) was gently mixed with 1 ml activating solution (80 mM 1-ethyl-3(3-dimethyl-aminopropyl)carbodiimide hydrochloride and 20 mM N-hydroxy succinimide) and then incubated at room temperature for 1 h. The PAA-TiO_2 suspension was concentrated with Amicon Ultra-4 (Millipore). A centrifuged sample (1 ml) of the activated PAA-TiO_2 suspension was mixed with 1 ml of the

recombinant protein solution (approximately 2 mg/ml), and the mixture was incubated at 4°C overnight. Anti-EGFR antibody (la) was used as the homing molecule, and BSA (Sigma-Aldrich, St Louis, USA) was used as the control. Subsequently, 1.0 ml of 0.1 M ethanolamine solution was added to block activated carboxyl residues and the mixture was incubated at 4°C for an additional 30 min.

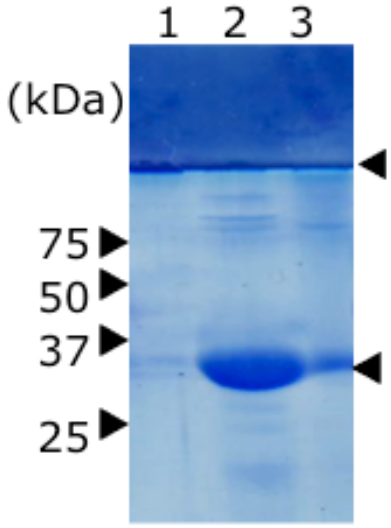

Figure S1. SDS-PAGE analysis of anti-EGFR antibody or anti-EGFR antibody (la)-modified TiO_2 nanoparticles. Lane 1: PAA-TiO_2; lane 2: PAA-TiO_2 and anti-EGFR antibody mixtures; lane 3: PAA-TiO_2/la

78

79 **Evaluation of the cell specific binding ability of anti-EGFR antibody-modified**
80 **TiO_2 nanoparticles using ELISA**

81

82 HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Nacalai
83 Tesque, Kyoto, Japan) containing 10% fetal bovine serum at 37°C under 5% CO_2. Cells
84 were grown in 96 well collagen coated 96 well plate (IWAKI Tokyo, Japan) with 2×10^5
85 cells/ml for 24 h. la, PAA-TiO_2 or PAA-TiO_2/la particles were added into the wells and
86 incubated additional 1h. After washing with serum-free DMEM three times, anti-6His
87 rabbit polyclonal antibody (final concentration is 2 ng/ml; Bethyl laboratories) was
88 added as a primary antibody. After incubation of 30 min, cells were washed as same
89 way, then HRP labeled goat anti-rabbit IgG (final concentration is 2 ng/ml; GE
90 healthcare) were added as secondary antibody. After further 30 min incubation, cells
91 were washed three times, and the amount of binding la or PAA-TiO_2/la was evaluated
92 using ECL plus detection system (GE healthcare).

93

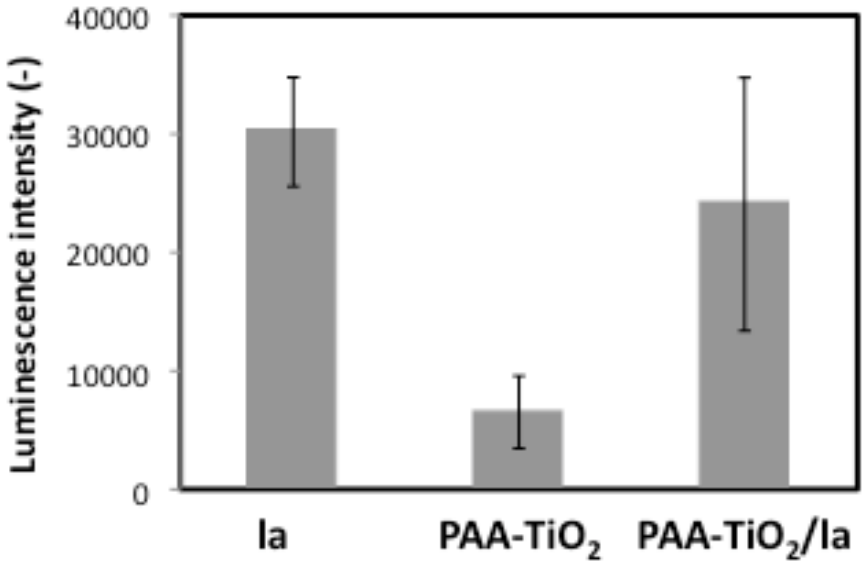

Figure S2 ELISA analysis of PAA-TiO_2/Ia binding ability to cells.

Evaluation of TiO_2 and PAATiO_2 nanoparticle radical generation

2-[6-(4'-Amino)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid (APF) (Sekisui Medical, Tokyo, Japan) was used to measure the amount of radicals generated from TiO_2 and PAA-TiO_2 nanoparticles by UV irradiation. These particles were dissolved in 20 mM HEPES buffer adjusted to 0.3, 0.03, 0.003, and 0.003 wt%. Then APF (10 mM) was added and UV irradiation was carried out using a UVB lamp (DNA-FIX, DF-312, Atto, Tokyo, Japan) with the peak at 312 nm. Fluorometric detection (ex. 495 nm and em. 515 nm) of radicals was carried out using a Wallac ARVO 1420 multilabel counter (Wallac OY, Turku, Finland). Radical generation was averaged over at least three independent experiments.

Cytotoxicity assay

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum at 37°C under 5% CO_2. Cells ware grown in 35-mm glass base dish (glass 12φ, Asahi Techno Glass Co., Tokyo, Japan) with 2×10^5 cells/ml for 24 h. PAA-TiO_2 or PAA-TiO_2/la particles were added and UV irradiation was applied. After irradiation, cell viability was immediately (or after further 24 h incubation with DMEM containing 10% FBS) evaluated using a Live/Dead Viability/Cytotoxicity kit (Invitrogen, Carlsbad, CA). In brief, dishes were washed with PBS and then 150 μl PBS-combined Live/Dead assay reagents (0.1 mM calcein AM and 2 mM EthD-1) were added. After incubation for 30 min at room temperature, the labeled cells were monitored under a fluorescence microscope (Keyence).