

Supplementary Information

Functionalised TiO₂ nanoparticles deliver oligo-histidine and avidin tagged biomolecules simultaneously into the cell

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Experimental Section

1. Materials:

Chemicals: Titanium (IV) oxide, anatase, nano powder was purchased from Sigma-Aldrich. Dry Toluene was purchased from Spectrochem. (3-glycidoxypropyl)trimethoxysilane (GOPTS) and Diisopropylcarbodiimide were purchased from Fluka. NiCl₂ Hexahydrate, N, N'-dimethyl formamide (DMF) were purchased from Merck. Trifluoroacetic acid was purchased from Acros Organic. Ethylene glycol and ammonia were purchased from RANKEM. All chemicals were used without further purification. FT-IR spectra were recorded on JASCO FT-IR 4200 model.

Biochemicals: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), β -casein, neutravidin and DMEM medium were purchased from Sigma-Aldrich. NHS-Biotin was purchased from Thermo Scientific. Diamino-polyethylene glycol and N₂H-PEG-NHBOC with MW 3000 Da were purchased from Rapp Polymer. Texas Red Avidin DCS was purchased from Vector laboratories. Tris-NTA and EGFP-His₁₀ were received as gift from Dr. Thomas Surrey's laboratory in EMBL, Heidelberg Germany. MDA-MB-231 Cell line was purchased from NCCS, Pune (India). Fetal bovine serum was purchased from Invitrogen.

2. Preparation of a stable colloidal suspension of titanium oxide nanoparticles:

A stable colloidal suspension of titanium oxide nanoparticles in milliQ water was prepared using the mixture of ethylene glycol and ammonia as dispersant materials. Ammonia and 5% wt of ethylene glycol was added to 100 ml of distilled water followed by the addition of 0.25 gm of titanium oxide nanoparticles. Then pH was adjusted to 9.5. The suspension was stirred for 30 min using magnetic stirrer. Then it was bath sonicated for 60 minutes and probe sonicated for 10 minutes¹. Thus stable colloidal suspension was maintained.

3. Preparation of dual functionalised titanium oxide (DFTO):

First titanium oxide nanopowder was silanised with 3-glycidoxypropyltrimethoxysilane (GOPTS).² 300 mg of TiO₂ was suspended in 10 mL of dry toluene under N₂. 1 mL of GOPTS was added to it and stirred for 4 hours under the refluxing condition of toluene. After completion of the reaction the solution was centrifuged at 10000 rpm for 10 minutes and the residue was washed 3 times by resuspension and centrifugation process using dry toluene.

Then it was dried under vacuum. For PEGylation, 50 mg H₂N-PEG-NH₂ and 50 mg H₂N-PEG-NHBOC were added to the 10 mg of silanised TiO₂ in 4 mL dry DMF and stirred for overnight under N₂ atmosphere. Next, the solution was centrifuged and the residue was washed for 3 times with dry DMF by sonication and centrifugation process. Next, 25 mg PEGylated TiO₂ was dissolved in 4 mL dry DMF and sonicated for 5 minutes under N₂ atmosphere. Then 6 mg of NHS-Biotin in 200 µL dry DMF was added into the solution of PEGylated TiO₂ and stirred at room temperature under N₂ for overnight. Excess solvent was evaporated and residue was treated with TFA for 6 hours to remove tertiary butyl group protection on amino group of PEG. Excess TFA was removed under vacuum and the residue was reacted with 25 mg of Tris-NTA in presence of 15 µL of DIC in dry DMF solution under N₂ atmosphere for 12 hour. Reaction mixture was centrifuged at 10000 rpm for 10 min and residue was washed with DMF following previously described method. Next after removal of excess DMF under vacuum, 3 mL of TFA was treated to the residue for 6 hours under N₂ at room temperature. Excess TFA was removed and residue was treated with milli-Q water followed by sonication for 5-10 min. Then it was centrifuged at 5000 rpm for 10 min and the residue was washed with Milli-Q water for 3-4 times by centrifugation and sonication process. Finally, dual functionalised TiO₂ (DFTO) solution in water was lyophilised and stored in 4 °C.

4. FT-IR Spectroscopy:

Dual functionalisation of TiO₂ was characterised by FT-IR spectroscopy.³ There are few characteristic vibration in the IR spectrum of TiO₂. The stretching band, centered at 1625 cm⁻¹ is Ti-O stretching and very broad band from 3000 cm⁻¹ to 3700 cm⁻¹ are due to -OH group adsorption. The absorbance peak at 1114 cm⁻¹ in TiO₂ is identified as C-OH stretching frequency. In case DFTO, the absorbance peak at 1678 cm⁻¹ is due to amide carbonyl stretching, which indicates functionalisation of TiO₂ (after PEGylation) with both biotin and Tris-NTA. This peak is also due to acid carbonyl stretching of Tris-NTA attached to PEGylated TiO₂. The lower stretching frequency of the acid group is due to hydrogen bonding. The broad band peak in the region 3000 cm⁻¹ to 3700 cm⁻¹ is due to -OH group, which is generated as a result of nucleophilic attack of amino group of H₂N-PEG-NH₂ and H₂N-PEG-NHBOC to the epoxide group of silanized TiO₂. The absorbance peaks in the region 2850 cm⁻¹ to 3000 cm⁻¹ are due to -CH stretching because of the presence of -CH₂ groups in DFTO.

5. High Resolution Transmission Electron Microscopy (HR-TEM):

Bare TiO₂ and DFTO was dissolved in milli Q water separately and sonicated for few minutes. 10 µL solutions of each were placed on two different carbon coated copper grids. After 1 min, excess fluid was removed and the grids were stained with 2% uranyl acetate in water. Excess staining solution was removed from the grid after two minutes and the samples were dried under vacuum. Then the samples were viewed using a JEOL JEM-2011 electron microscope operating at 200 kV. From HR-TEM images, it was observed that there is a change in the surface morphology of TiO₂ nanoparticles after functionalisation. In case of bare TiO₂ nanoparticles, we observed well organised line pattern like structure, but in case of

DFTO, this line pattern was absent. This result indicates functionalisations driven morphological changes of titanium oxide.

6. Particle size measurement:

The size of DFTO nanoparticle was measured by Malvern particle size analyser (MALVERN ZETASIZER NANO ZS, 173° back scattering). The size was measured in DMEM (Sigma) after 5 minutes of probe sonication. Average particle size of the DFTO nanoparticle was found around 195 nm (PDI-0.373).

7. Fluorescence Microscopic Imaging:

DFTO was equilibrated with 10 mM NiCl₂ solution using a mechanical shaker for 15 min followed by centrifugation at 10000 rpm for 10 min. Residue was washed with Milli-Q water for 3 times with intermittent sonication and centrifugation. Next, DFTO was equilibrated with BRB80 at 4 °C and followed by incubation with β -casein by gentle shaking for 15 min at 4 °C. Excess β -casein was removed by centrifugation at 10000 rpm for 10 min at 4 °C followed by washing with BRB80 through sonication and centrifugation method at 4 °C (2 times). DFTO was incubated with 500 nM Texas Red Avidin DCS by gentle shaking using mechanical shaker for 15 min at 4 °C. Removal of excess dye and washing was done following previously described method. Next, Avidin dye bound DFTO was incubated with 500 nM EGFP-His₁₀ in BRB80 by gentle shaking on mechanical shaker for 15 min at 4 °C. Excess EGFP-His₁₀ was removed by centrifugation at 10000 rpm and washed with BRB80 by sonication/resuspension method at 4 °C (2 times). Finally, 20 μ L of dual fluorophore bound DFTO solution in BRB80 was loaded on ethanol cleaned microscopic glass slide and on top of that solution another 22 mm/22 mm coverslip was placed for making a sandwich. Sandwich was sealed with VALAC and imaged using 488 and 561 nm laser through an IX-81 fluorescence microscope (Olympus) with a x60 objective (Olympus; Hamburg, Germany) and an Andor iXon3 897 Camera.

8. Reversible binding of EGFP-His₁₀ onto DFTO nanoparticles:

In this experiment, dual fluorophore binding with DFTO nanoparticles was done following above described method. Dual fluorophore bound DFTO was incubated with 500 mM Imidazole on a mild shaker for 10 min at 4 °C. Mixture was centrifuged and Imidazole was removed add fresh 500 mM Imidazole was loaded again and repeat previous step for three times. Now, DFTO nanoparticles were washed with BRB80 by resuspension and centrifugation method at 4 °C for 4-5 times. DFTO nanoparticles were imaged as above mentioned method. In 488 nm laser light we found very weak green signal on DFTO nanoparticles but, at 561 nm laser light we found strong red signal on same area of the DFTO nanoparticles which clearly indicates that imidazole washed out the EGFP-His₁₀. Now DFTO was washed with BRB80 to get rid of imidazole. Next, EGFP-His₁₀ was reloaded following previously described method. Finally, imaging was done as previously described method and

observed strong signal both in red and green channel indicates that EGFP-His₁₀ was bounded again on DFTO nanoparticles (Figure S1).

9. Polymerization or nucleation of microtubules from DFTO nanoparticles:

DFTO nanoparticles were equilibrated with NiCl₂ and β -casein as mentioned in the above procedure. Next, it was incubated with 0.3 μ M neutravidin in BRB80 by gentle shaking on mechanical shaker for 15 min at 4 °C. Excess neutravidin was removed by centrifugation at 10000 rpm and washed with BRB80 by centrifugation and sonication method at 4 °C. This neutravidin loaded DFTO nanoparticles solution in BRB80 was used during the preparation of microtubules.

Mixture A: 0.25 μ L Alexa568 Tubulin (15 mg/mL) + 0.25 μ L Biotin Tubulin (15 mg/mL) + 2 μ L Tubulin (27 mg/mL) + 2 μ L neutravidin loaded DFTO nanoparticles solution + 2.3 μ L BRB80 + 0.2 μ L GTP-Mg (25 mM).

Mixture B: 180 μ L of BRB 80 and 0.36 μ L Taxol (20 μ M).

Mixture **A** and **B** were warmed at 37 °C for 30 minutes and then 45 μ L mixture **B** added into the mixture **A** and mixed carefully. Then the mixture was centrifuged for 8 min at 12000 rpm at 37 °C. Then the supernatant was carefully taken out and coloured pellet was resuspended and incubated with 500 nM EGFP-His₁₀ in BRB80 with taxol by gentle shaking for 5 minutes under warm condition. Excess EGFP-His₁₀ was removed by centrifugation at 10000 rpm and washed with warm BRB80 containing 20 μ M taxol. Then 100 μ L of BRB80 with 20 μ M taxol was added to it. Finally, imaging was done as previously described method. In 488 nm laser, we saw the green coloured nanoparticles and in the 561 laser red coloured microtubules was grown from that green coloured nanoparticles. It was further confirmed by the overlay image which clearly indicates immobilisation and nucleation of microtubules from DFTO nanoparticles.

10. Control Experiment:

Control experiment was carried out with PEGylated TiO₂. PEGylated TiO₂ was treated with Ni⁺² and β -casein as previously described method. Both the fluorophores were immobilized following the same procedure as above (similar method as described in dual fluorophore binding experiment). Finally, imaging was done as previously described method and PEGylated TiO₂ was observed in bright field, 488 and 561 nm laser light separately. No fluorescent signal was observed at 488 and 561 nm laser light. It clearly states that fluorophore binds only through functional groups.

11. Cytotoxicity study:

Cytotoxicity of the DFTO nanoparticles were evaluated by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction.⁴ MDA-MB-231 cells were seeded at a density of 10000 cells per well in a 96-well plate for 18-24 hours before dual functionalised titanium oxide treatment. MDA-MB-231 cell was treated with various concentration of dual functionalised titanium oxide (stock concentration was 0.5 mg/mL) in DMEM medium

containing 10% fetal bovine serum for 24h and 48 h in different 96-well plate. Following the termination of experiment, cells were washed and promptly assayed for viability using MTT. Results were expressed as percent viability = $[\text{A550 (treated cells)} - \text{background} / \text{A550 (untreated cells)} - \text{background}] \times 100$. The results show non cytotoxic nature of DFTO nanoparticles.

12. Cellular uptake studies:

Cellular uptake of the His-tagged EGFP and Avidin texas red loaded dual functionalised titanium oxide was studied. MDA-MB-231 Cell was seeded at a density of 100000 cells per well in a 6-well plate for 18-24 hours before His-tagged EGFP and Avidin texas red loaded DFTO nanoparticles treatment. MDA-MB-231 Cell was treated with His-tagged EGFP and Avidin texas red loaded DFTO nanoparticles solution in 3 mL serum free DMEM medium (Concentration of His-tagged EGFP and Avidin texas red loaded DFTO nanoparticles in medium is 5 µg/ml) for 4h. After 4h the medium was discarded and 3 mL of DMEM medium containing 10% fetal bovine serum was added to well and left for 24h. Cellular uptake studies were performed in MDA-MB-231 cell at 37 °C in presence of 5% CO₂. After 24 hour addition of His-tagged EGFP and Avidin texas red loaded DFTO nanoparticles, the cells were washed with phosphate buffer saline and the live MDA-MB-231 cells were viewed with an NIKON inverted microscope (Model Ti-U).

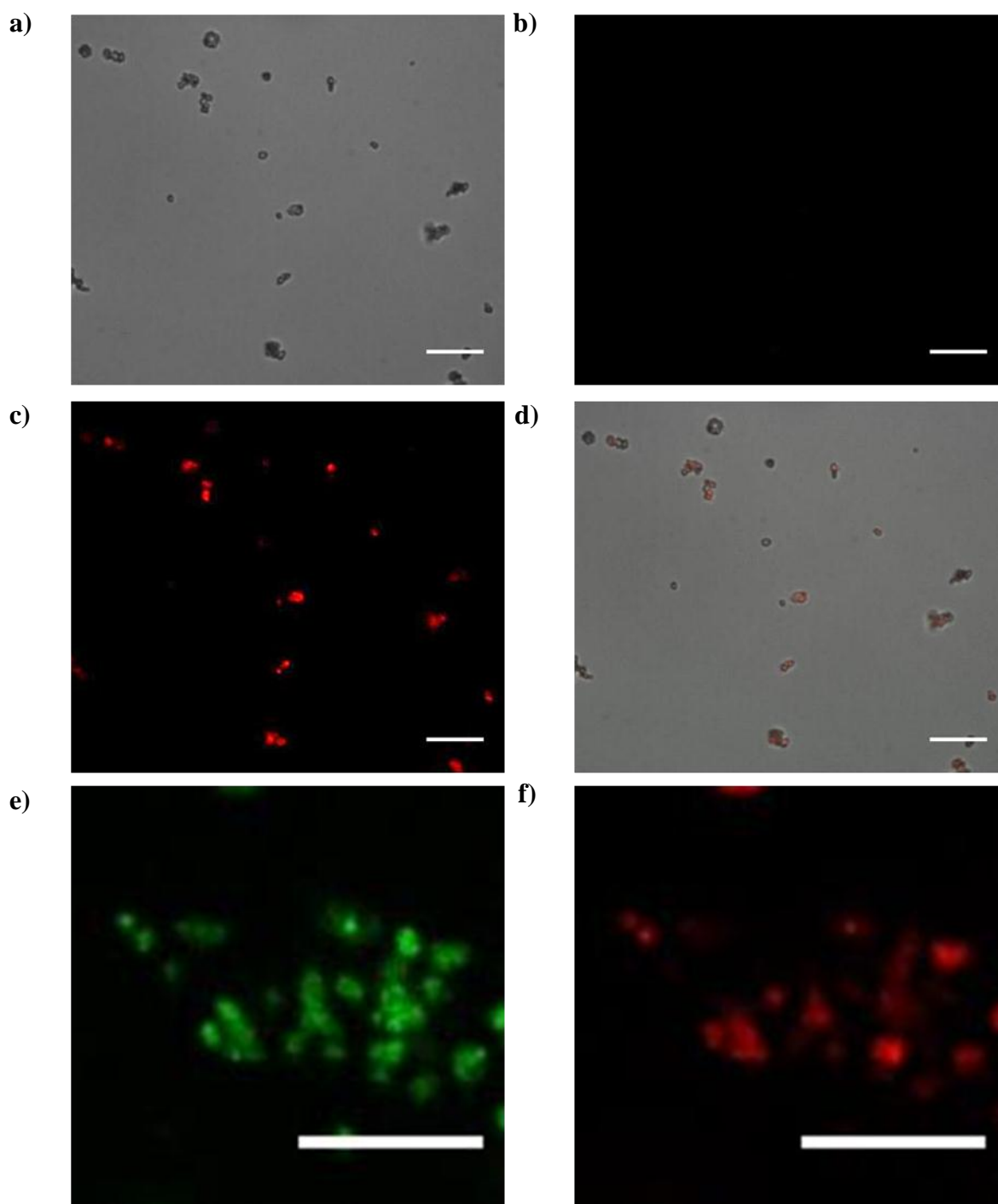


Figure S1. Fluorescence microscopic images of reversible mode of EGFP-His₁₀ binding on DFTO nanoparticles. (a) Black coloured, EGFP-His₁₀ and avidin texas red dye loaded DFTO nanoparticles in DIC mode after imidazole treatment; (b) no green colour was observed on EGFP-His₁₀ and avidin texas red dye loaded DFTO nanoparticles after imidazole treatment in 488 channel; (c) red coloured EGFP-His₁₀ and avidin texas red dye loaded DFTO nanoparticles, after imidazole treatment in 561 channel; (d) Overlay image of DFTO nanoparticles after imidazole treatment.; (e) green colour reappears in 488 channel after loading EGFP-His₁₀ again; (f) red coloured DFTO nanoparticles after loading again EGFP-His₁₀ in 561 channel. Scale bar corresponds to 10 μ m.

References:

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