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Supporting Information

Barstar:Barnase – A Versatile Platform for Nanodiamond Bioconjugation

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Reaction 1: 140-nm LND aqueous colloidal solution was obtained from Academia Sinica, Taiwan. 140-nm LND aqueous colloid (300 µL, 0.1% w/v), with *pH* adjusted to 4.5 using hydrochloric acid, was activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (2.3 µL, 10 mg mL^{-1}) and sulfo-N-hydroxy succinimide (sNHS) (49.2 µL, 10 mg mL^{-1}) for 15 min in a bath sonicator at room temperature. Sonication was necessary to prevent flocculation of LNDs due to the presence of EDC and sNHS, which occurred otherwise. Excess EDC and sNHS were removed from the activated LND colloid by centrifugation at 4300 g for 3 min, and re-suspension of the pellet in distilled deionised water (DDW) [In order to obtain a control sample of the non-activated LNDs, i.e. LND+Bs (see text for cross-comparison of LND-Bs and LND+Bs), the above steps were carried out without adding EDC and sNHS]. DDW was preferred over any buffer because of the possible flocculation. For binding reaction, Bs solution (200 µL, 2.5 mg mL^{-1}) was prepared in phosphate buffer with 20 mM NaCl (PB20, *pH* 7.2) to which activated LNDs were added drop-wise to counter aggregation by promoting LND-Bs reaction pathway, rather than LND+LND interaction. This binding reaction between the activated LNDs and Bs was carried out for 2 h under sonication at room temperature, after which the un-reacted barstar was removed by centrifugation (4300 g for 3 min) and re-suspension of the LND-Bs pellet in PB20. This washing procedure was repeated four times, with a dilution factor of 1:40 in each wash. Sonication during the LND activation reaction and drop-wise addition of the activated LNDs to the buffered Bs-solution represented an enabling modification of the conventional protocol, which was essential in preventing LND aggregation.

XPS Analysis: The freshly synthesized LND-Bs sample was washed with PB20, under sonication, 7 times, with a dilution factor of 1:15, by centrifugation at 4300 g for 3 min to remove free Bs, with the unchanged zeta-potential confirmed. 10 µL of this sample was drop-dried on a clean, plasma-etched glass slide for the XPS analysis. To discriminate between covalent bonding and adsorption of LND-Bs and LND+Bs complexes, PB20 colloidal *pH* was varied from *pH* 4 to *pH* 10.8 during the washing procedure.

Reaction 2(A)-Preparation of LND-Bs:Bn-EGFP and analysis: LND-Bs was mixed with an excess of Bn-EGFP for 30 min at room temperature. After the reaction, the solution was centrifuged at 14000 g for 5 min. The supernatant (SN_1) was collected, and the pellet was re-suspended in PB20. This washing procedure was repeated the 2nd and a 3rd time yielding supernatants SN_2 and SN_3, respectively, and the resultant pellet was re-suspended in PB20, referred to as LND-Bs:Bn-EGFP.
Samples for FCM were prepared by mixing LND-Bs:Bn-EGFP (10 µL) and poly-vinyl-alcohol solution in water (10 µL, 1% w/v). In some occasions, fluorescent beads were added to the sample to facilitate finding the sample plane during FCM imaging. This mixture was dropped on a cover slip mounted on a vacuum-sealed spinning head, and spun at 1000 rpm for 3 min. The cover slip was then covered with a glass slide and sealed for microscopy observations.

Reaction 2(B)-Preparation of LND-Bs:Bn-nAu and TEM imaging: LND-Bs was mixed with an excess of pre-conjugated barnase-nanogold sub-unit (Bn-nAu) for 60 min under sonication, followed by three centrifugation (at 16000 g for 3 min) and resuspension in PB20 to remove the free Bn-nAu. The final pellet (LND-Bs:Bn-nAu) was resuspended in PB20. A TEM sample was prepared by deposition of a drop of LND-Bs:Bn-nAu colloid on a carbon film, supported by a copper grid that was dried under vacuum. TEM images were acquired using a PHILIPS CM10 system at an accelerating voltage of 100 kV. An example image of the LND-Bs:Bn-nAu is shown in the Fig. 1.

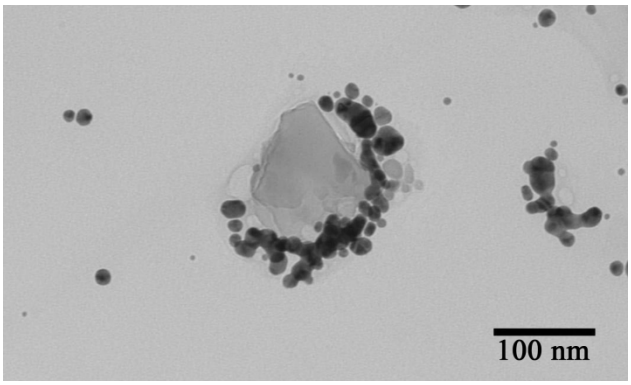

Fig. 1 TEM image of LND-Bs:Bn-nAu complex.

Cellular labeling with LND-Bs:Bn-EGFP: The Chinese hamster ovary (CHO-K1) cells were grown, in F12 Ham's media supplemented with FBS (10%) and G418 (400 μg mL^{-1}) (Invitrogen Inc.), to approximately 80% confluence and seeded on an 8-well slide on the day before the experiment. On the day of experiment, the cells in the 8-well slide were incubated with LND-Bs:Bn-EGFP solution in PBS [with additional calcium chloride (0.9 mM), magnesium chloride (0.5 mM) and D-glucose (20 mM) and bovine serum albumin (0.1% w/v)] for 40 min at 37 °C under 5% CO_2]. The cells were subsequently washed with PBS and then fixed with freshly prepared paraformaldehyde (3.7%) in PBS for 20 min at room temperature. After fixation, the cells were washed again with PBS (twice) and sealed with a cover slip with PBS as mounting media.
Imaging was carried out using FCM, with a 488-nm laser beam of approximately 0.5-mW power with an oil-immersion objective lens (100× N.A. 1.4). For spectral acquisition, images were taken sequentially at different emission wavelengths (spectral window, 25 nm) under the same excitation. The images were processed with Image J and Adobe Creative Suite 4.

Barstar (Bs) production: The mutant barstar C40A (hereafter referred to as barstar or Bs) was expressed in *E. coli* strain HB101, freshly transformed with the plasmid pMT643 (a kind gift of Dr. R. W. Hartley), and isolated from the cleared bacterial lysate. Nucleic acids were removed by precipitation with polyethyleneimine (0.04%); and proteins were fractionated with ammonium sulfate. The fraction of 65-75% saturation of ammonium sulfate was purified by gel filtration using Sephadex G100 SuperFine column, (C16/100) equilibrated with Tris-HCl (20 mM), NaCl (20 mM), EDTA (2 mM), DTT (2 mM), Tween-20 (0.05%), *pH* 8.0. The 105-120 mL fraction of the eluate was applied onto Q-Sepharose HiTrap FF, washed with the same buffer and eluted in Tris-HCl (20 mM), EDTA (2 mM), DTT (2 mM) and glycerol (10%), *pH* 8.0 with a linear gradient of 0-0.5 M NaCl, 0.05-0.1 mL min^{-1}. 2 mL fractions were collected and analyzed by SDS-PAGE.

Barnase (Bn) production: Wild-type barnase (hereafter referred to as barnase or Bn) was produced in *E. coli* strain TG-1 freshly transformed with the plasmid pMT413 (a kind gift of Dr. R. W. Hartley), encoding the barnase with phoA signal peptide under the control of *tac* promoter, and the barstar under the control of its own constitutive promoter. Barnase was extracted and purified from culture medium following the method by Hartley with modifications[1]. Culture medium containing barnase was chilled on ice supplemented with 5% acetic acid and clarified by centrifugation at 8000 rpm, 5 min, 4 ˚C. The supernatant was mixed with phosphocellulose P11 (2 g of dry weight per liter of supernatant) equilibrated with NaOAc (20 mM), *pH* 4.5, gently stirred on ice for 2 h, and the supernatant was removed by decantation. The suspension of P11 was washed (by decantation) three times with NaOAc (1 L of 20 mM), *pH* 4.5 and packed into a column. The column was equilibrated with NaOAc (20 mM), *pH* 4.5, and barnase was eluted with a linear gradient of NH_4OAc (20-800 mM), *pH* 8.0, in EDTA (10 mM) and mercaptoethanol (0.1%). Barnase with minor concomitant proteins were eluted at 400-600 mM NH_4OAc. This barnase eluate was diluted ten-fold with NH_4OAc (20 mM), EDTA (10 mM), mercaptoethanol (0.1%), *pH* 5.0, and applied to a sulfopropylsepharose column. The column was equilibrated with NaOAc (20 mM), *pH* 5.0, and barnase was eluted with NaOAc (l00 mM), EDTA (10 mM), mercaptoethanol (0.1%), *pH* 6.0.

Barnase-EGFP (Bn-EGFP) production: To produce Bn-EGFP fusion protein, we used *E. coli* strain HB101 transformed with the plasmid pEGFP-bn[2]. This plasmid encoded EGFP-barnase fusion protein supplied with His_5 tag under the control of *tac* promoter, and the barstar under the control of its own natural promoter. The cell extract containing the complex of EGFP-barnase with barstar was adsorbed to a Ni^{2+}-NTA Sepharose and the fusion protein was purified by denaturation with urea (8 M) and NaCl (2 M) to remove barstar inhibitor and refolded on the column. Finally, the targeting protein was eluted with imidazole (120 mM).

LND surface chemistry characterization:The LND samples for this work were purchased from Academia Sinica, Taiwan. According to the company[3], LNDs were surface-functionalized with carboxyl groups by strong oxidative acid treatment in concentrated H_2SO_4-HNO_3 (9:1, v/v) at 75 °C for 3 days, subsequently in NaOH (0.1 M) aqueous solution at 90°C for 2 h, and

finally in HCl (0.1 M) aqueous solution at 90 °C for 2 h. The resulting carboxylated/oxidized FNDs were separated by centrifugation, rinsed extensively, and resuspended in deionized water.

The manufacturers presented FTIR analysis of the as-received LND surface structure of the acid-treated sample that exhibited abundance of COOH-groups, as shown in Fig. 2. We relied on this data in our communication of the EDC/sNHS reaction (Reaction 1).

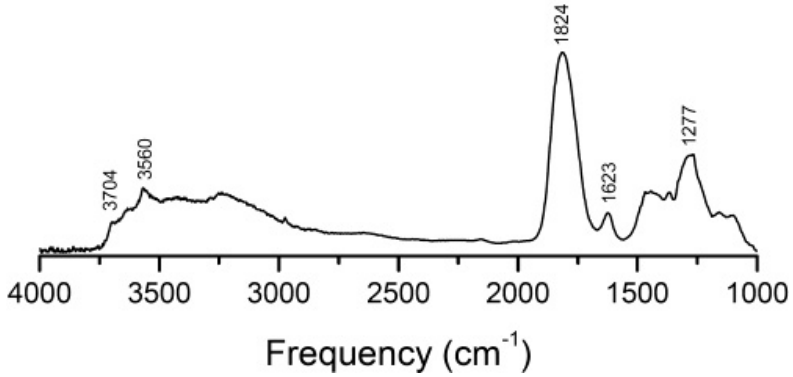

Fig. 2 FTIR spectrum of the as-received acid-treated LND sample. Two spectral features at 3560 cm^{-1} and 1824 cm^{-1} point to the presence of COOH-surface groups, C-O, and C=O stretching frequencies, respectively. Reproduced from Ref. 3. with ACS copyright permission.

Bn-nAu conjugation: The nanogold (nAu) represented a ZrO_2-core gold nano-shell of 20 nm in diameter (produced in the Institute of Problems of Chemical Physics, Moscow, Russia). First, thiol-PEG-COOH (500 Da, 0.57 µg) was added to nAu aqueous suspension (250-µL, 5×10^{12} units per mL), and allowed to react for 60 min in a bath sonicator at room temperature. Excess of thiol-PEG-COOH was removed by centrifugation at 16000 g for 3 min, three times. The resultant nanoparticles, covered with thiol-PEG-COOH, were activated with EDC (2.9 µg) and sulfo-NHS (4.6 µg) in MES buffer (1 mL) (*pH* 5), aided by sonication for 20 min. Subsequently, the particles were washed, resuspended in Bn-solution (1 mL, 100 mg mL^{-1}) in PBS (*pH* 7.2) and incubated in a bath sonicator for 60 min. Then nanoparticles were allowed to stay overnight at +4 °C. The free Bn-excess was removed by centrifugation and re-suspension of the pellet resuspended in PBS (*pH* 7.2), three times.

Estimation of a number of EGFP molecules per LND particle: Fluorescence of the diluted LND-Bs:Bn-EGFP colloid was recorded using Fluorolog Tau3 system (JY Horiba, Edison, NJ). A xenon lamp (bandpass-filtered at 480 nm) and 532-nm laser, were chosen for efficient excitation of EGFP and LND, respectively. The EGFP signal from the conjugate solution was compared with those of known concentrations to obtain its concentration to be 0.8 nM. Similarly, the LND concentration was estimated to be 1.9×10^{-6} w/v. This yielded an EGFP per LND number ratio of 1300, i.e. 1300 EGFP molecules attached on 140-nm LND surface.

Straightforward geometrical considerations yielded a limiting value of ~7000 Bs molecules per LND particle on the assumption of uniform surface population of the barstar molecules. Bs diameter was estimated to be 3 nm, based on the assumption of globular protein conformation, and LND sphere total area was taken as 6×10^{4} nm^2.

Bleaching of EGFP under FCM: This experiment was carried out to reconcile the EGFP per LND fluorescence signal ratio that was >> 1 as acquired by the Fluorimeter and ~1, using FCM. We tested a hypothesis of EGFP photobleaching under high-intensity focused laser radiation at 488 nm employed in FCM.

The EGFP sample for FCM was prepared similar to LND-Bs:Bn-EGFP, explained previously. The cover slip was sealed onto a glass slide, and placed under FCM. A 488-nm laser beam of 1-mW power was focused on the sample surface by a 100× oil-immersion objective lens and raster-scanned in a square-shaped pattern across the sample repeatedly to induce photobleaching of EGFP. Image area was zoomed out and the photobleached area was observed (see Fig. 3).

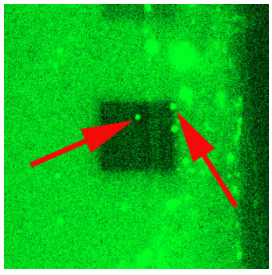

Fig. 3 Fluorescence confocal microscopy image of EGFP embedded in a PVA polymer film, which was photobleached within a square area (dark green color). The rest was unbleached (intense green). Bright circular spots marked by arrows were identified as the auxiliary fluorescent micro-beads.

35-nm LND-Bs conjugation: 35-nm HTHP nanodiamonds (obtained from the same manufacturer, Academia Sinica, Taiwan) were conjugated with Bs following the same Reaction 1 protocol, as described in the main text. The zeta-potential measurements yielded values of -50±4, -44±3 and -31±1 mV for LND(DDW), LND (PB20) and LND-Bs(PB20) respectively. The zeta-potential variation indicated that the EDC/NHS reaction modified the surface charge of the HTHP nanodiamond by, presumably, populating the surface with Bs-molecules.

Supporting Information References

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