Fluorometric assay of integrin activity with a small-molecular probe that senses the binding site microenvironment

Toru Komatsu, Aoi Takeda, Kenjiro Hanaoka, Takuya Terai, Tasuku Ueno, Yukio Tada, Tetsuo Nagano and Yasuteru Urano

Methods

Chemical synthesis and characterization. General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, Aldrich Chemical Co., and Invitrogen, and were used without further purification. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a JEOL JNM-LA300 instrument. δ values are in ppm relative to tetramethylsilane (TMS). Mass spectra (MS) were measured with a JEOL JMS-T100LC AccuTOF (ESI).

Assay preparation. Soluble integrin $\alpha_V\beta_3$ was purchased from U.S. Biological and a stock solution was prepared according to the manufacturer's instructions. Aliquots of the stock solution were prepared and stored at -80˚C. Each aliquot was used for a single assay to avoid repeated freeze-thaw cycles. Probes and inhibitors were prepared as DMSO stock solutions of 10 mM, and diluted to the required concentrations with the assay buffer. Assay was performed in sodium phosphate buffer (100 mM, pH 7.4) containing 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 1% CHAPS.

Fluorometric assay. Probe and proteins were mixed in assay buffer in wells of a 384-well plate (Greiner 784076), and the plate was incubated for 30 min at 25˚C. Fluorescence intensity or fluorescence polarization assay was performed with a plate reader PHERAstar (BMG Labtech), under appropriate filter conditions.

Fluorometric assay for Stern-Volmer plotting and calculation of quantum yields. Fluorescence spectra were measured by F-7000 (HITACHI). Absorbance spectra were measured by UV-2550 (SHIMADZU). Quantum yields were calculated from the below equation. Φ_{FL} = 0.85 of fluorescein in 0.1 M NaOH (aq.) was used as a standard.

$$\emptyset_{fl}^{\text{sample}} = \emptyset_{fl}^{\text{std}} \frac{Abs^{\text{std}}}{Abs^{\text{sample}}} \frac{\int F^{\text{sample}}}{\int F^{\text{std}}} \frac{n^{\text{sample}^2}}{n^{\text{std}^2}} \frac{I^{\text{std}}}{I^{\text{sample}}}$$

Absorption assay. Probe and proteins were mixed in assay buffer in 0.5 mL plastic tubes, which were incubated for 30 min at 25˚C. The solution was loaded on a NanoDrop 1000 (Thermo Fisher Scientific) and absorbance spectra were recorded.

Cyclic Voltammetry. Analyzer: Als/chi 600A electrochemical analyser (three electrode system); Counter electrode: Pt wire; measurement electrode: glassy carbon or Pt; reference electrode: Ag/Ag^{+}.

Calculation of probe binding with proteins. Based on the K_d value calculated from binding assay, the concentration of the probe-protein complex and its proportion with respect to the total probe concentration (P_{bound}) under various conditions were estimated according to the following equations. Calculated P_{bound} under each condition is given in figure legends.

$$Protein_{free} + Inhibitor_{free} \leftrightarrows Complex$$

$P_{free} = [Protein_{free}]$, $P_0 = [Protein_{total}]$, $I_{free} = [Inhibitor_{free}]$, $I_0 = [Inhibitor_{total}]$, $C = [Complex]$

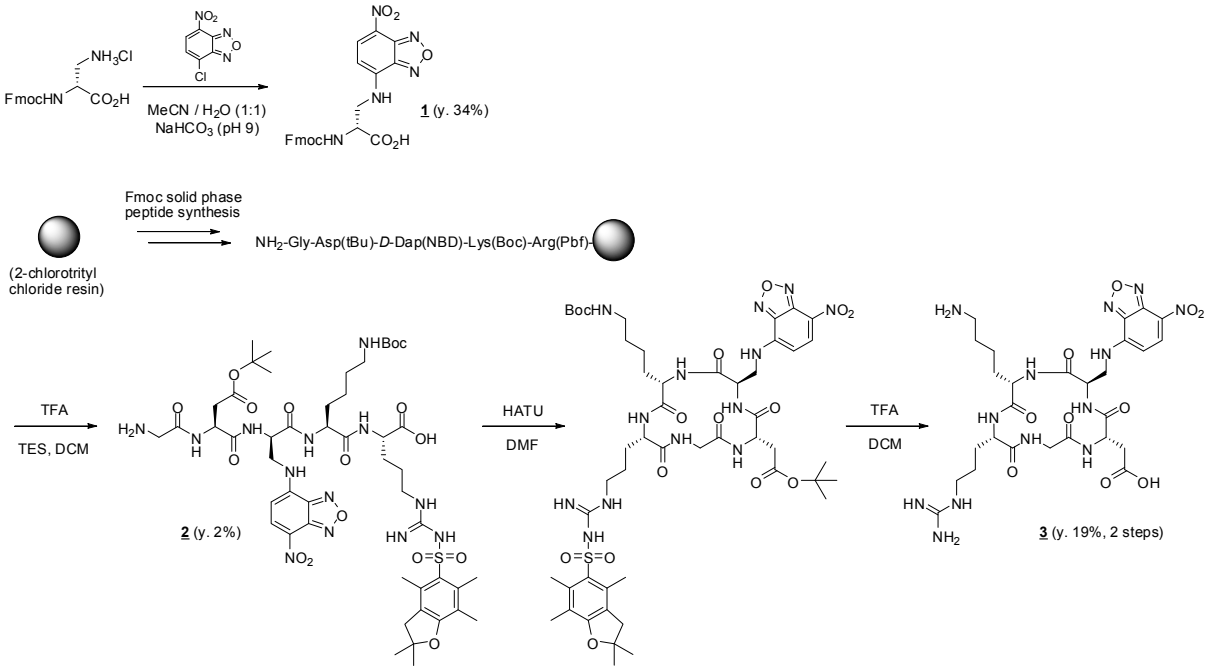
$$K_d = \frac{P_{free} \cdot I_{free}}{C} = \frac{(P_0 - C)(I_0 - C)}{C}$$

$$C = \frac{(K_d + P_0 + I_0) - SQRT((K_d + P_0 + I_0)^2 - 4P_0I_0)}{2}$$

$$P_{bound} = \frac{C}{I_0}$$

Docking Study. Docking study was performed using MMFF94x (Merck molecular force field). The calculation was based on the reported co-crystal structure of c(RGDfV) and integrin $\alpha_V\beta_3$ (PDB: 1L5G), by replacing the structure of c(RGDfV) with that of cRGD-NBD.

Synthesis



Scheme S1. Preparation of NBD-bearing amino acid and the cyclic RBG-based fluorescent probe.

Preparation of Fmoc-*D*-Dap(NBD)-OH (1). Prepared according to the reported synthetic route for Fmoc-*S*-Dap(NBD)-OH[1]. Fmoc-*D*-Dap-OH/HCl (363 mg, 1.0 mmol) was mixed with NBD-Cl (220 mg, 1.1 mmol) in 50 mL CH_3CN/H_2O 1:1 containing $NaHCO_3$ (pH 9). The reaction mixture was stirred for 5 hr, then acidified (pH 3) with 2 N HCl, and the product was extracted with ethyl acetate three times. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and evaporated. The crude product was purified by column chromatography (silica gel; dichloromethane (DCM)-5% methanol-0.5% acetic acid) to afford pure **3** (270 mg, 55%). ^{1}H-NMR (300 MHz, DMSO-d_6): δ 3.58 (br, 2H); 4.18 (t, 1H, J = 6.6 Hz); 4.30 (d, 2H, J = 7.3 Hz); 4.42 (m, 1H); 6.48 (d, 1H, J = 8.8 Hz); 7.2-7.9 (m, 8H); 8.50 (d, 1H, J = 8.8 Hz); 9.37 (br, 1H); 13.10 (br, 1H). ^{13}C-NMR (75 MHz, DMSO-d_6): δ 43.97; 46.53; 52.90; 65.71; 99.52; 120.09; 120.13; 125.07; 126.96; 127.57; 127.61; 137.91; 140.69; 140.72; 143.43; 156.06; 170.37. HRMS (ESI^-): *m/z* Calcd. for $(M-H^+)^-$; 488.1206, Found; 488.1192.

[1] *Tetrahedron Letters* **2000**, *41*, 6063-6066.

General procedure for preparation of protected linear RGD peptides. Protected linear peptides were synthesized by an automatic peptide synthesizer Syro I (Biotage) using standard protocols of fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis with 2-chlorotrityl chloride resin (0.1 mmol reaction site) (Novabiochem, USA). The resin beads were washed 3 times with 3 mL dichloromethane (DCM) and the peptide was cleaved from the resin with 2 mL DCM containing 1% trifluoroacetic acid (TFA) and 1% triethylsilane (TES) for 2 hr. After filtration, the filtrate was azeotroped with toluene and peptides were purified

by reverse-phase HPLC. The *N*-terminal of the linear peptides was chosen as glycine in order to achieve higher yield in the subsequent cyclization.

Preparation of linear Gly-Asp(*t*Bu)-*D*-Dap(NBD)-Lys(Boc)-Arg(Pbf)-OH (2). Linear peptide for cRGD-NBD (**3**) was prepared from Fmoc-Gly-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-*D*-Dap(NBD)-OH, Fmoc-Lys(Boc)-OH and Fmoc-Arg(Pbf)-OH (2.3 mg, 2%).
LRMS: m/z = 1133 $(M+H^+)^+$.

Preparation of linear Gly-Asp(*t*Bu)-*D*-Tyr-*D*-Dap(NBD)-Arg(Pbf)-OH (4). Linear peptide for cRGD-5'-NBD (**5**) was prepared from Fmoc-Gly-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-*D*-Tyr(*t*Bu)-OH, Fmoc-*D*-Dap(NBD)-OH and Fmoc-Arg(Pbf)-OH. LRMS: m/z = 1123 $(M+H^+)^+$.

Preparation of linear Gly-Asp(*t*Bu)-*D*-Tyr-Lys-Arg(Pbf)-OH (6). Linear peptide for c(RGDyK) (**7**) was prepared from Fmoc-Gly-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-*D*-Tyr(*t*Bu)-OH, Fmoc-Lys(Boc)-OH and Fmoc-Arg(Pbf)-OH. LRMS: m/z = 1102 $(M+H^+)^+$.

General procedure for preparation of cyclic RGD peptide probes. Linear protected peptide (0.002 mmol) was dissolved in 2 mL *N*,*N*-dimethylformamide (DMF), and HATU (7.7 mg, 0.02 mmol) was added. The reaction mixture was stirred for 2 hr, and formation of the cyclized product was confirmed with ESI-MS. After evaporation of the mixture, 2 mL TFA and 1% TES were added, and stirring was continued for 2 hr. The mixture was evaporated and the crude product was purified by reverse-phase HPLC.

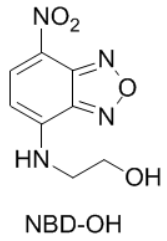
Preparation of cyclic-Arg-Gly-Asp-*D*-Dap(NBD)-Lys (cRGD-NBD, 3). Prepared from **2** (0.3 mg, 19%). LRMS: m/z = 706 $(M+H^+)^+$.

Preparation of cyclic-Arg-Gly-Asp-*D*-Tyr-*D*-Dap(NBD) (cRGD-5'-NBD, 5). Prepared from **4** (0.2 mg, 1% in 2 steps). LRMS: m/z = 741 $(M+H^+)^+$.

Preparation of cyclic-Arg-Gly-Asp-*D*-Tyr-Lys(Alexa594) (c(RGD-Alexa594), 7). Compound **6** was cyclized, and the purified cyclic peptide (0.5 mg) was conjugated with Alexa594 *N*-succinimidyl ester (2.5 mg) in 100 mM sodium borate buffer (pH 9.5) for 2 hr. The product was purified by preparative HPLC (y. trace). LRMS: m/z = 1324 $(M+H^+)^+$.

Supplementary Data

Fluorescence character of NBD-modified amines. In order to study the fluorescence character of NBD-modified amines and the effect of probe interaction with the hydroxyphenyl group of tyrosine, NBD-OH was prepared according to the literature[2]. NBD-modified 2,3-diaminopropionic acid (Dap) is expected to exhibit similar fluorescence properties.



NBD-OH

	ET_{30}[3]	Abs. λ_{max}	Em. λ_{max}	Φ_{fl}
in PBS	63.1	479	531	0.025
in MeOH	55.4	465	520	0.212
in DMSO	45.6	477	528	0.247
in MeCN	45.1	467	515	0.439
in DCM	40.7	455	506	0.438
in Dioxane	36	452	511	0.439

Figure S1. Fluorescence character of NBD-OH in various solvents.

[2] *J. Am. Chem. Soc.*, **2013**, *135*, 18651-18658

[3] *Chem. Rev.*, **1994**, *94*, 2319-2358

	$\lambda_{ex.}$ (nm)	$\lambda_{em.}$ (nm)	$\Delta E_{0,0}$ (eV)	E_{red} (eV vs. Ag/Ag^+)	$-(E_{red} + \Delta E_{0,0})$
NBD-OH	477	546	2.42	-1.26	-1.16

Figure S2. Results of cyclic voltammetric analysis of NBD-OH.

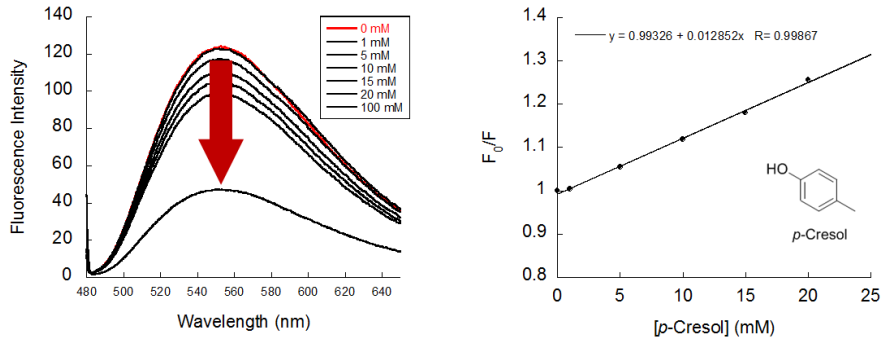


Figure S3. (Left) Fluorescence spectra of 3 μM NBD-OH in PBS (pH 7.4) containing 0.3% DMSO and 0, 5, 10, 15, 20, and 100 mM *p*-cresol. Excitation wavelength for fluorescence spectra was 480 nm. (Right) Stern-Volmer plot of fluorescence quenching by *p*-cresol. In the concentration range of 0-20 mM, there was no detectable change in the absorbance spectrum of the NBD fluorophore.

[Comment]

The measured value of E_{red} of NBD-OH indicates that the electron density of the NBD fluorophore was lower than those of commonly used fluorophores, including fluorescein and BODIPY, which are known to be fluorescently quenchable by tyrosine based on the PeT mechanism[4]. Therefore, quenching efficiency between NBD and tyrosine can be expected to be relatively strong. Also, from **Figure S3**, the K_{SV} of NBD fluorescence quenching by *p*-cresol, a Tyr analog, was calculated to be 12.9 (/M). From the reported fluorescence lifetime of NBD-modified amines (1.0 (ns))[5], k_q was calculated to be 1.29×10^{10} (/M/s), which is greater than the electron transfer quenching rate constant between tyrosine and fluorescein (2.4×10^{9} /M/s) or BODIPY-FL (4.0×10^{9}/M/s) [2], as expected. The value is high enough to account for photoinduced electron transfer (PeT)-based fluorescence quenching when NBD fluorophore and tyrosine are in close proximity.

[4] *Bioconjugate Chem.* **2003**, *14,* 1133-1139.

[5] *J. Photochem. Photobiol. A*, **1993**, *70*, 229-243.

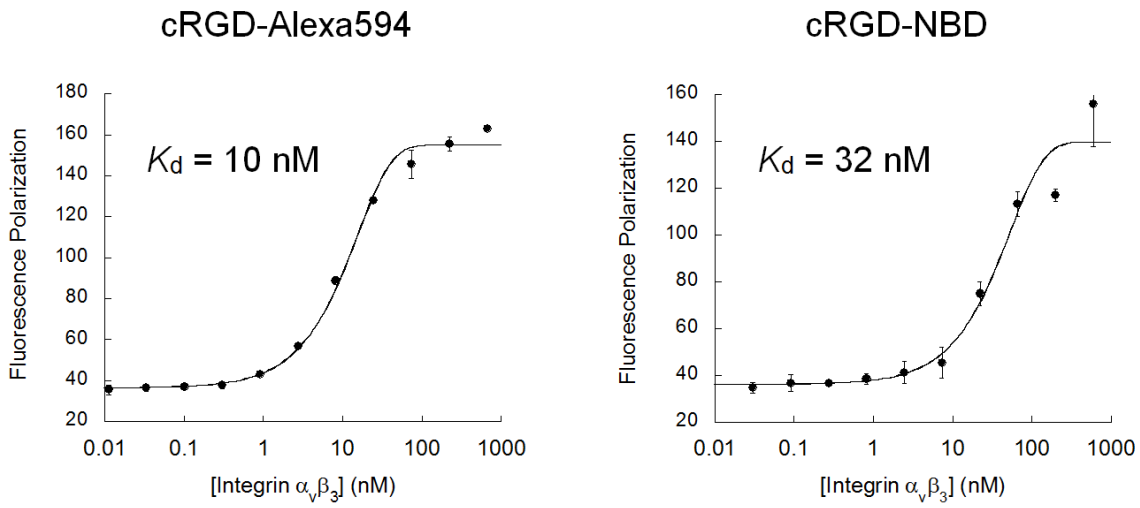


Figure S4. Calculation of K_d values of cRGD-Alexa594 (left) and cRGD-NBD (right) (10 nM) based on fluorescence polarization measurement in the presence of different concentrations of integrin.

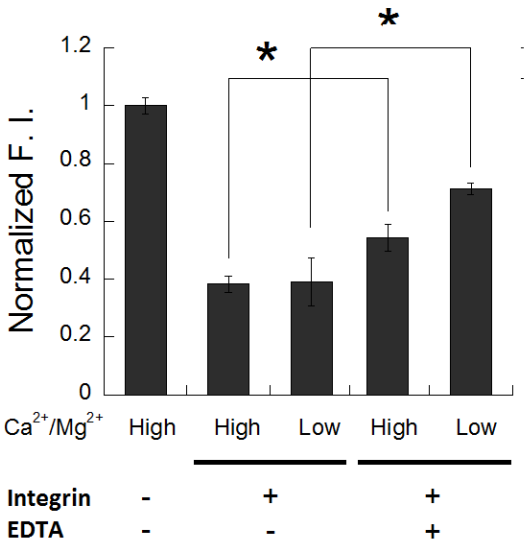


Figure S5. Normalized fluorescence intensities of cRGD-NBD (10 nM) in the presence and absence of soluble integrin $\alpha_V\beta_3$ (100 nM) in PBS (pH 7.4) containing 1% CHAPS and high or low concentrations of Ca^{2+} and Mg^{2+} (high: 1 mM $MgCl_2$ and 2 mM $CaCl_2$, low: 0.1 mM $MgCl_2$ and 0.2 mM $CaCl_2$). The effect of ethylenediaminetetraacetic acid (EDTA, 10 mM), which acts as a chelator of secondary ions, was also tested. n = 4. Error bar represents S.D. * $P < 0.05$.

Importance of 4'-modification for fluorescence quenching of NBD-fluorophore. In order to examine the importance of proximity of the NBD fluorophore to Tyr[122], we prepared another cyclic RGD peptide-based probe, named cRGD-5'-NBD. The 5'-substituent of cyclic RGD peptide is known to be directed outside of the integrin binding site, so interaction of the fluorophore with the binding site is expected to be weak.

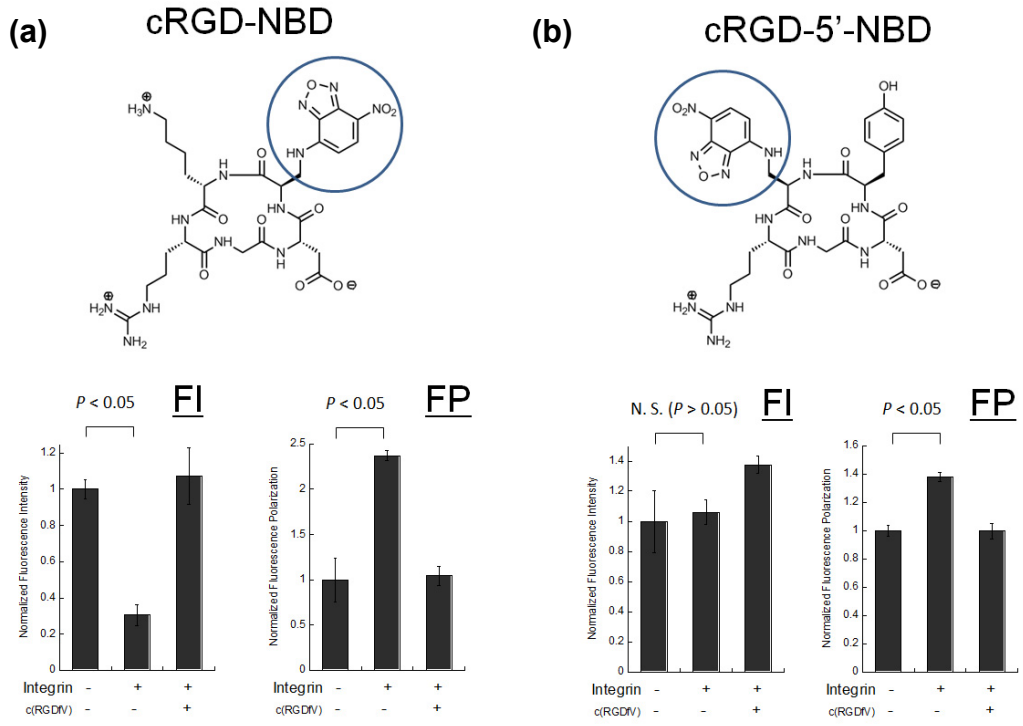


Figure S6. Fluorescence change of cRGD-NBD or cRGD-5'-NBD upon binding with integrin $\alpha_V\beta_3$. (a) Structure of 4'-modified peptide probe cRGD-NBD and the result of binding assay of cRGD-NBD (10 nM) with soluble integrin $\alpha_V\beta_3$ (100 nM). The values of fluorescence intensity (FI) and fluorescence polarization (FP), normalized to the signals without integrin, are shown. Concentration of c(RGDfV) was 1000 nM. n = 4. *P* values were calculated using Student's t-test. (b) Structure of 5'-modified peptide probe cRGD-5'-NBD and the result of binding assay of cRGD-5'-NBD (10 nM) with soluble integrin $\alpha_V\beta_3$ (100 nM). The values of fluorescence intensity (FI) and fluorescence polarization (FP), normalized to the signals without integrin, are shown. n = 4.

Study of fluorescence change due to non-specific binding to a protein surface. As a model of non-specific binding to a protein surface, we examined the effect of bovine serum albumin (BSA) on the fluorescence of cRGD-NBD.

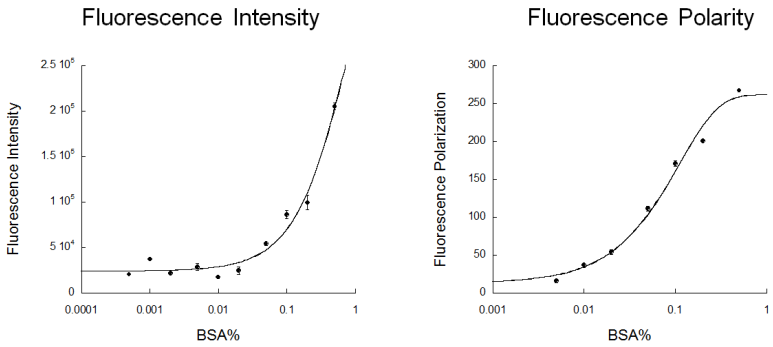


Figure S7. Fluorescence intensity (left) and fluorescence polarization (right) of 10 nM cRGD-NBD with bovine serum albumin (BSA) in PBS (pH 7.4) containing 1 mM $MgCl_2$, 2 mM $CaCl_2$. Error bars represent S.D. (n = 4).

[Comment]

NBD fluorescence is known to be sensitive to the polarity of solvents, and we found that non-specific binding to BSA resulted in a slight increase of the fluorescence, as shown in **Figure S7**. However, fluorescence decrease, as observed in the case of binding to integrin, was not observed in the case of non-specific binding, and appears to be specifically due to NBD's interaction with the Tyr-122 side chain at the binding site of integrin.