Biophysical Journal, Volume 97

Supporting Material

APPLICATION OF FLUORESCENCE SPECTROSCOPY TO QUANTIFY SHEAR-INDUCED PROTEIN CONFORMATION CHANGE

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APPLICATION OF FLUORESCENCE SPECTROSCOPY TO QUANTIFY SHEAR-INDUCED PROTEIN CONFORMATION CHANGE

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Table of Contents

I. Methods

Expression and purification of dimeric VWF

Dimeric VWF (ΔPro-VWF) was expressed in stably transfected Chinese Hamster Ovary (CHO) cells (1). Cell culture protocol and purification methods were improved from that described previously (1) and this resulted in improved yield and purity as described here. The CHO cells were initially expanded in Dulbecco's modified eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) up to confluence in T150 tissue culture flasks. Following this, the cells were washed with phosphate buffered saline (PBS) to remove serum containing media, and the culture media were changed to protein-free ProCHO-AT (Lonza, Basel, Switzerland). After 72 h, the cell culture supernatant was collected and centrifuged at 1300 g for 10 min to remove any cells and cell debris. A two-step procedure was then used to purify dimeric VWF from culture media. In the first step, anion exchange chromatography (4 mL Fractogel® TMAE column, Merck, Gibbstown, NJ) was performed using a fast protein liquid chromatography (FPLC) system from Amersham Biosciences/GE Healthcare Biosciences (Piscataway, NJ). During this step, the anion exchange chromatography column was initially equilibrated with 20 mM Tris buffer pH 7.4. Cell culture supernatant was diluted 2-fold with 20 mM Tris buffer (pH 7.4) and was run through the column. Following several column washes with 20 mM Tris (pH 7.4) to remove non-specifically bound protein, the bound dimeric VWF was eluted using 20 mM Tris (pH 7.4) containing 400 mM NaCl. In the second step, 1.5 mg rabbit anti-human VWF polyclonal antibody (Dako, Carpinteria, CA) was dissolved in 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3, and this was immobilized on 5 mL NHS-activated HP immunoaffinity column (GE Healthcare, NJ) following manufacturer's instructions. Dimeric VWF eluted following anion exchange chromatography (first step) was applied to this immunoaffinity column. Following the capture of dimeric VWF to the immunoaffinity matrix, several column volumes of 20 mM Tris containing 300 mM NaCl (pH 7.4) were applied to remove non-specifically bound protein. Bound dimeric VWF was then eluted using 0.1 M glycine at pH 1.5. The 1 mL fraction containing the dimeric VWF was neutralized using 120 μL of 1 M Tris, pH 7.4.

Platelet binding to immobilized VWF: A1 domain function of purified and recombinant protein

Polyclonal rabbit anti-human VWF antibody was covalently immobilized on 5.7 μm polybead carboxylated microbeads (Polysciences, Warrington, PA) using carbodiimide chemistry (2). Once prepared, these beads were stored in HEPES buffer containing 0.1% Human Serum Albumin (HSA) at a concentration of 20 x 10^6 /mL. For immobilization of proteins onto these beads, 60 μL of the antibody coated beads at 20 x 10^6 /mL were incubated with 10 μg/mL of multimeric or dimeric VWF for 20 min at room temperature. Excess VWF was removed by spinning and resuspending the beads in HEPES buffer containing 0.1% HSA. The resulting beads are termed 'multimeric-VWF beads' and 'dimeric-VWF beads'.

Normal human platelets in platelet rich plasma (PRP) were isolated from blood obtained from healthy human volunteers by brief centrifugation at 150 g for 12 min (3). Isolated platelets were labeled with Phycoerythrin (PE) labeled anti-PECAM-1 (CD31, platelet endothelial cell adhesion molecule-1) mAb WM59 (BD Pharamingen, San Jose, CA). In a typical run, 10 μL of PRP containing ~300 × 10^6 platelets/mL were incubated with 3 μL mAb for 10 min at room temperature prior to experimentation.

For the adhesion assay, multimeric/dimeric VWF-beads (2 x 10^6 /mL) and anti-CD31PE-labeled platelets (15 x 10^6 /mL) were diluted in HEPES buffer containing 0.1% HSA and 1.5 mM

$CaCl_2$. This suspension was subjected to mixing at a shear rate of 1863 /s in a cone-and-plate viscometer (Haake VT550, 0.5° cone angle, 5 cm diameter). 5 μL samples collected at 1 min were diluted in 95 μL HEPES buffer and immediately read in a FACSCalibur Flow Cytometer (BD Biosciences). Characteristic forward- versus side- scatter (FSC vs SSC) profiles were used to discriminate between platelets and beads. Platelet/CD31-PE fluorescence associated with the bead population was used to quantify the percent of beads with bound platelets. Control runs were performed using anti-VWF beads without any bound VWF. In additional runs, platelet GpIb-VWF binding interactions were blocked using 20 µg/ml blocking antibody against GpIb, clone AK2 (Millipore/Chemicon, Billerica, MA).

Factor VIII binding to VWF

The binding of multimeric and dimeric VWF to Factor VIII was determined using ELISA. 2 μg/ml of anti-VWF monoclonal antibody (AVW-1, GTI Diagnostics, Milwaukee, WI) diluted in Hepes buffer was absorbed overnight at 4ºC onto Maxisorp plates (Nunc/ThermoFisher, Rochester, NY). Wells were blocked with 3% BSA (w/v) for 2 h at room temperature. Following this, 2 μg/mL of multimeric or dimeric VWF were incubated in wells for 1 h. 2 μg/mL Recombinate Antihemophilic Factor VIII (Baxter, Deerfield, IL) dissolved in HEPES buffer was then added to the plate. Sheep HRP-conjugated anti-Factor VIII antibody (1 μg/ml, Enzyme Research, Swansea, UK) was used for detection of Factor VIII bound to plate, and OPD (*o*-Phenylenediamine) reagent from Sigma-Aldrich was used as the substrate. Absorbance measurements were performed at 495 nm using a plate reader from Molecular Devices (Sunnyvale, CA). In competitive blocking assays, 2 μg/mL Factor VIII was pre-incubated with 25 μg/mL anti-Factor VIII mAb ESH4 (American Diagnostica, Stamford, CT) for 30 min in HEPES buffer at 37°C prior to addition of Factor VIII to the VWF-bearing wells in the above assay.

References:

1. Singh, I., H. Shankaran, M. E. Beauharnois, Z. Xiao, P. Alexandridis, and S. Neelamegham. 2006. Solution structure of human von Willebrand factor studied using small angle neutron scattering. J Biol Chem 281:38266-38275.
2. Xiao, Z., H. L. Goldsmith, F. A. McIntosh, H. Shankaran, and S. Neelamegham. 2006. Biomechanics of P-selectin PSGL-1 bonds: shear threshold and integrin-independent cell adhesion. Biophys J 90:2221-2234.
3. Shankaran, H., P. Alexandridis, and S. Neelamegham. 2003. Aspects of hydrodynamic shear regulating shear-induced platelet activation and self-association of von Willebrand factor in suspension. Blood 101:2637-2645.

II. Supplemental Figures

Figure S1
Themistou et al.

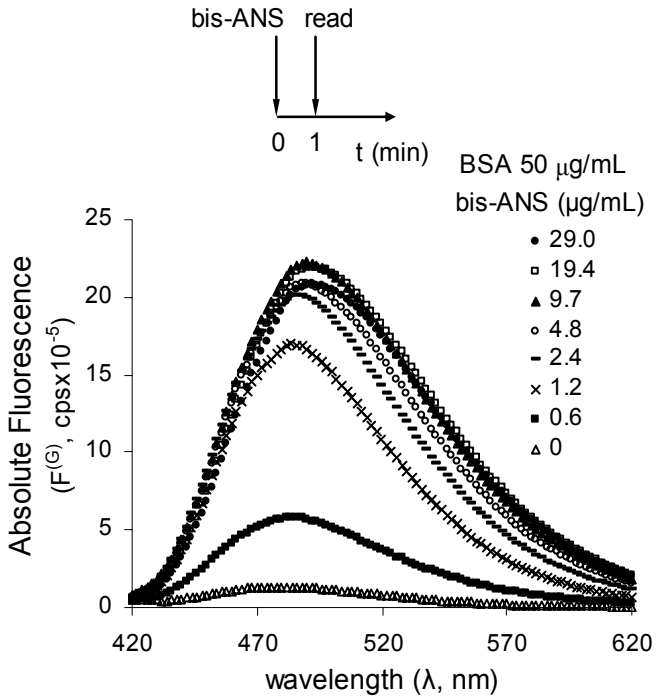

Figure S1. Absolute fluorescence when various concentrations of bis-ANS were added to 50 μg/mL BSA.

Figure S2
Themistou et al.

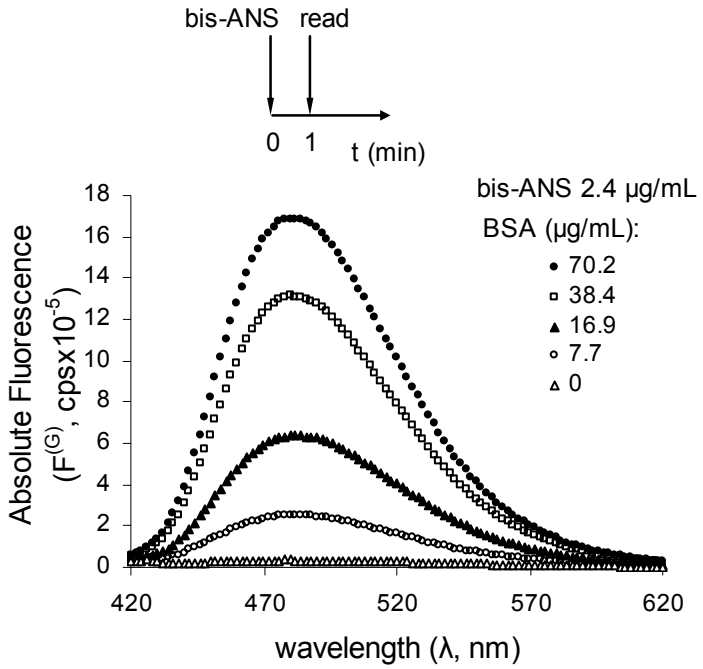

Figure S2. Absolute fluorescence when various concentrations of BSA were added to 2.4 μg/mL bis-ANS.

Figure S3
Themistou et al.

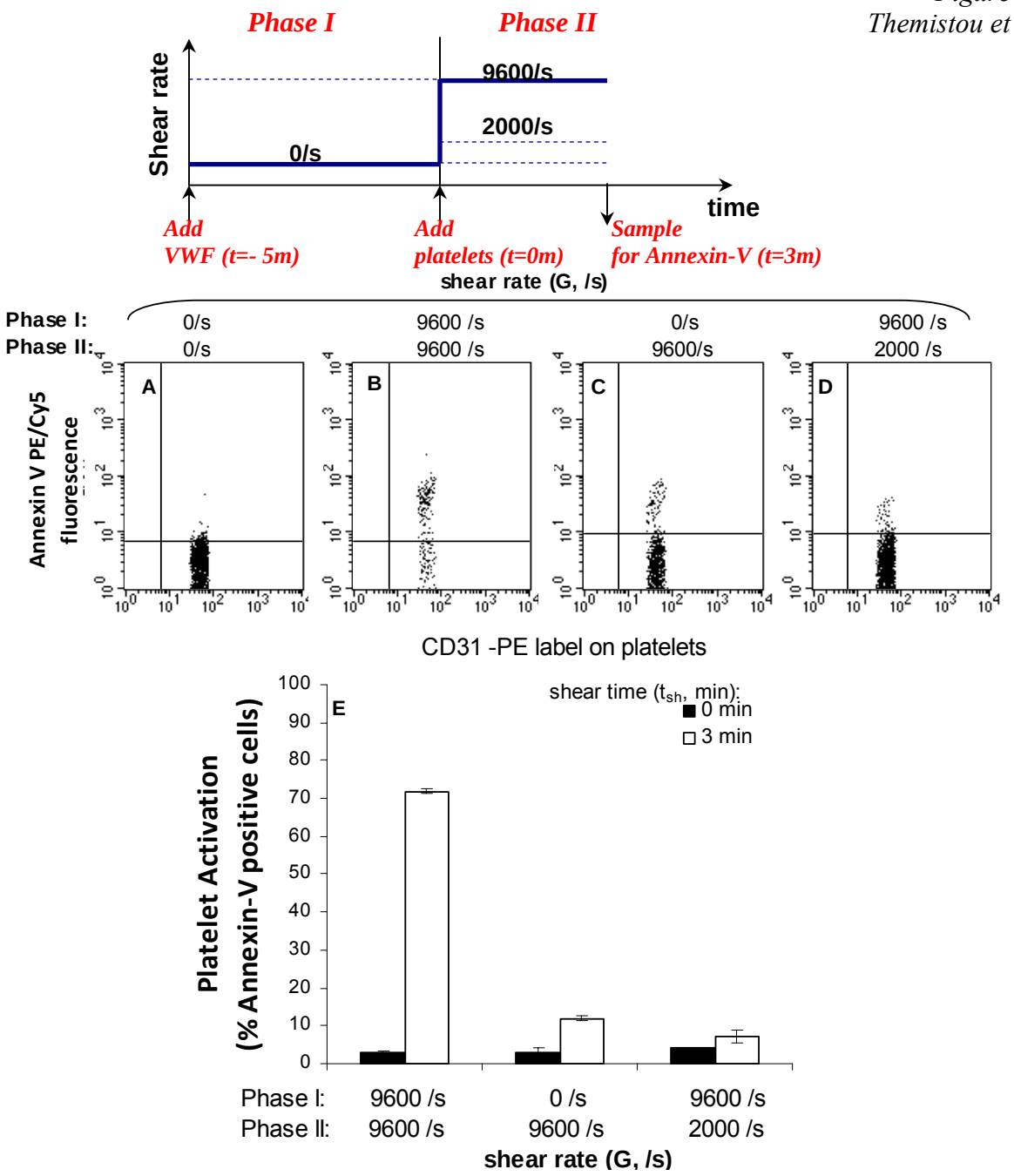

Figure S3. Effect of shearing VWF. Experiments were performed in two phases as illustrated in the schematic. In the first phase (denoted Phase I), VWF (no platelets) was subjected to shear in the viscometer at either 9600 /s for 5 min or the protein was kept under static conditions at 0 /s. 30×10^6/mL CD31-PE labeled isolated human platelets were added to 30 µg/mL VWF at the start of the second phase (denoted Phase II), and this mixture was subjected to a variety of shear rates as indicated. After 3 min., Annexin V-PE/Cy5 binding to phosphatidylserine exposed on platelet surface was measured using flow cytometry as described earlier (Shankaran *et al.*, 101: 2637-45, 2003). Bold blue line in schematic corresponds to protocol used in panel C. **A-D**. Flow cytometry dot plots show that the fraction of active platelets (greater than baseline Annexin-V binding) varies depending on the shear protocol in Phase I and II. **E.** Summary of data show that pre-shearing VWF in Phase I dramatically increases the extent of platelet activation in Phase II compared to runs where VWF was either unsheared in Phase I or when the level of shear in Phase II is low (2000 /s).

Figure S4
Themistou et al.

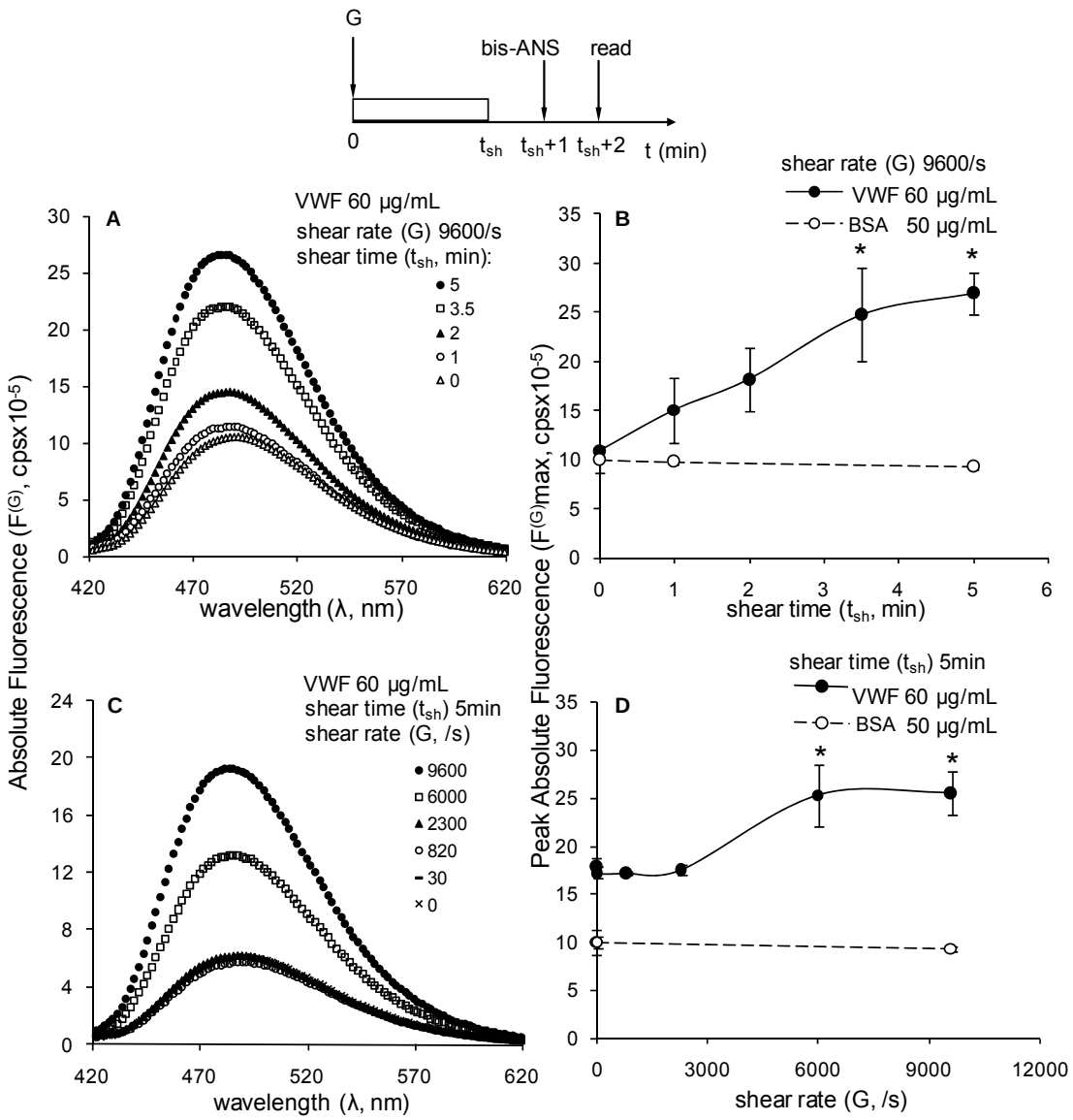

Figure S4. Absolute fluorescence value plots show that bis-ANS binding to VWF subjected to fluid shear is significantly higher (*$p < 0.05$) than VWF binding to unsheared protein at time ≥ 3.5 min and G ≥ 6000 /s, identical to that described in Figure 3 of the main manuscript.

Figure S5
Themistou et al.

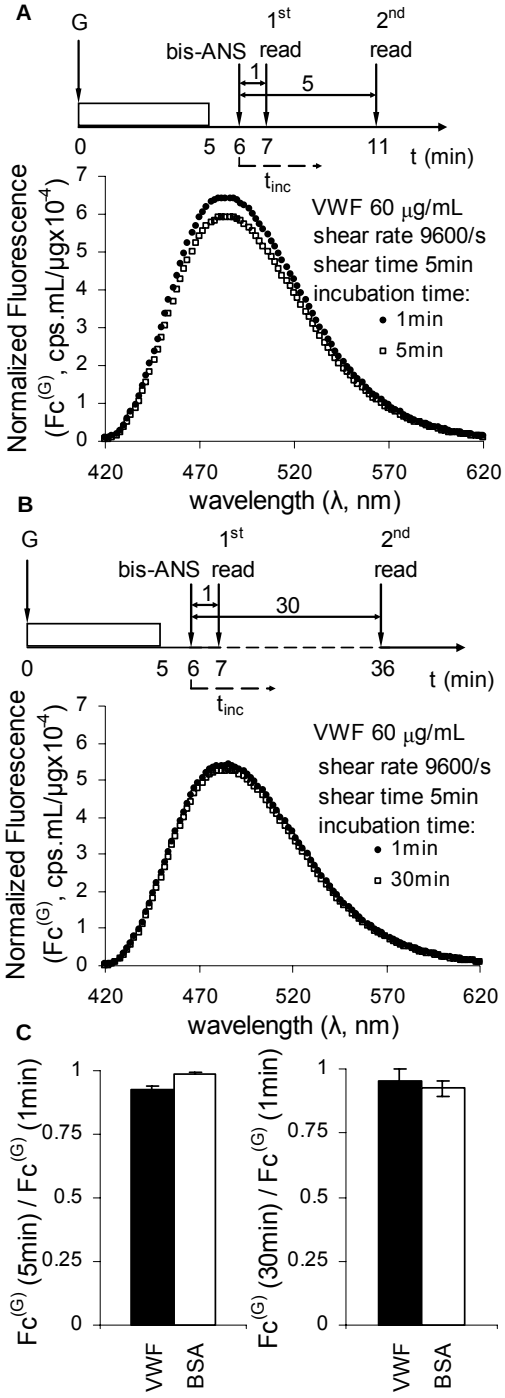

Figure S5. Time of bis-ANS addition. Fluid shear was applied to 60 μg/mL multimeric VWF using a cone-plate viscometer at 9600 /s for 5 min. Bis-ANS was added 1 min after shear stoppage and fluorescence spectra were recorded either at **A.** 1 and 5 min, or at **B.** 1 and 30 min, after probe addition (t_{inc}) as illustrated in the schematics. Normalized fluorescence ($F_c^{(G)}$) data are presented. **C.** Ratio of peak $F_c^{(G)}$ value at either 5 or 30 min compared to that at 1 min. Peak $F_c^{(G)}$ was calculated at λ = 484-488 nm for multimeric VWF and 480-484 nm for BSA. Error bars represent S.D. for 3 independent experiments with either multimeric VWF or BSA.

Figure S6
Themistou et al.

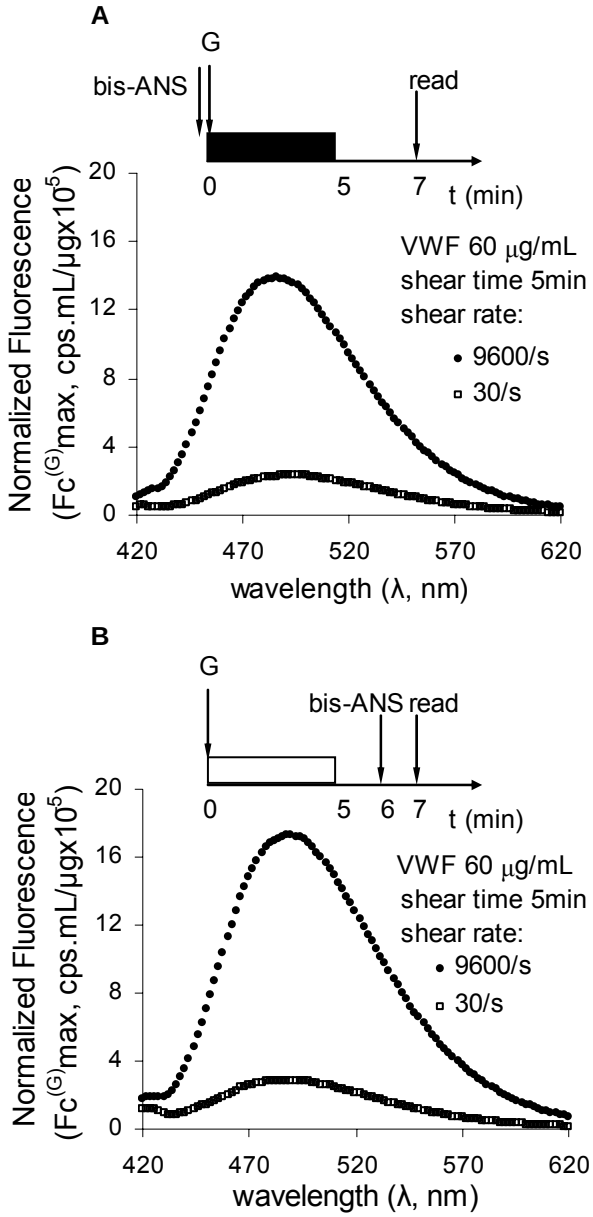

Figure S6. Bis-ANS addition to VWF before shear application. Fluid shear was applied to 60 µg/mL multimeric VWF using a cone-plate viscometer at 9600 /s and 30 /s for 5 min. Bis-ANS was added either **A.** before shear application, or **B.** 1 min after shear stoppage. In both cases, fluorescence was measured at 7 min as illustrated in the schematics.