Ligand binding alters dimerization and sequestering of urokinase receptors in raft-mimicking lipid mixtures

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EPI-Analysis of uPAR distribution in the bilayer

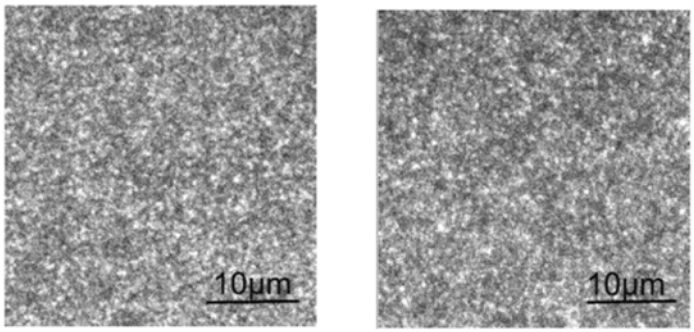

Fig. S1: Epifluorescence micrographs of uPAR distribution (marked by anti-DDK antibody, concentration 0.0012mol% related to lipids in DOPC (A) and DOPC + 35mol% CHOL (B) bilayers. The micrographs show the largely homogeneous distribution of uPAR (no large-scale uPAR aggregation) in the bilayer regardless of the absence or presence of CHOL.

Methodology: Analysis of uPAR concentration in the bilayer

To identify the concentration of uPAR in the bilayer, TRITC-DHPE was first applied as a calibration standard. In this case, several DOPC bilayer samples were made with different, well-defined concentrations of TRITC-DHPE. Here three bilayers were characterized for each given TRITC-DHPE concentration point and the distribution and brightness of dye-lipid in the bilayer was determined using CS-XY scans and confocal fluorescence intensity analysis, respectively. To achieve statistical significance, the fluorescence intensities of about 15 random areas per TRITC-DHPE concentration were determined, averaged, and background-corrected. Next, in a follow up experiment, we made bilayer samples, incubated uPAR of three different concentrations, and added an equimolar ratio (relative to uPAR) of dye-labeled anti-DDK antibody. Independent FCS autocorrelation analysis of free Alexa 555 dye molecules and Alexa 555-labeled antibody in solution showed that the average ratio of dye-to-antibody is 1.1:1. The antibody incubation time of 2 hours was identical to typical experiments of uPAR analysis in the bilayer. To allow background correction, dye-labeled anti-DDK antibody was also added to uPAR-free bilayer samples in the same amounts as used for uPAR-containing bilayers. The intensity analysis on uPAR-containing and

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uPAR-free bilayers followed similar protocols, as described for TRITC-DHPE. The results from these experiments are illustrated in Fig. S2.

On the basis of the TRITC-DHPE calibration data, the known dye-to-antibody-ratio (from FCS autocorrelation analysis), and the known amounts of added uPAR/antibody, one is able to determine the amount of antibody-labeled uPAR in the bilayer. Specifically, this can be achieved by analyzing the slopes of the linear fits of uPAR and TRITC-DHPE data in Fig. S2. The slope of the TRITC-DHPE calibration curve is $(1.07\pm0.08)\times10^{5}$ (kHz/mol%) whereas the slope for uPAR is $(0.92\pm0.05)\times10^{5}$, which indicates that 86±6% of uPAR incorporated correctly into the bilayer (uPAR count rates in Fig. S2 are corrected by a factor of 0.91 to take into account independently determined dye-to-antibody ratio). In our typical experiments, the incubated amount of uPAR was 1.3×10^{-11} mol. If all protein added were incubated into the bilayer and antibody-labeled, we would have a molar concentration of uPAR (relative to lipid) of 1.1×10^{-3} mol% (shown as solid marker of uPAR intensities in Fig. S2). From the calibration curve, the overall signal from dye-labeled antibody (added in equimolar ratio to uPAR) is $(1.0\pm0.09)\times10^{-3}$ mol%, corresponding to the amount of 86% of antibody-labeled uPAR in the bilayer.

Fig. S2 also provides valuable information about the potential extent of antibody-mediated uPAR crosslinking. As two cross-linked uPAR molecules would share the same single fluorescent antibody probe, antibody-mediated crosslinking would cause the apparent concentration of uPAR to be decreased. Fig. S2 shows that assuming no-crosslinking, 86% of the uPAR were incorporated and properly labeled on average at all three different concentrations of uPAR probed ($R^2 = 0.99$). This leaves at most 14% of potentially antibody-crosslinked uPAR if one assumes 100% of added uPAR was incorporated into the bilayer and antibody-labeled, an unlikely scenario. In other words, the results from Fig. S2 indicate that antibody-mediated uPAR crosslinking is rather insignificant. This finding is plausible if one considers that uPAR and anti DDK-antibodies were added in equimolar amounts.

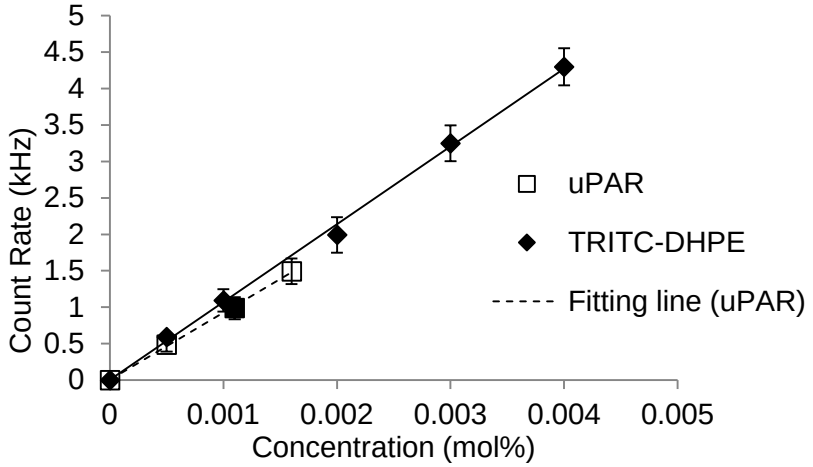

Fig. S2: Calibration of fluorescence intensity by comparing different concentrations of TRITC-DHPE (diamond) and antibody-labeled uPAR in the bilayer (square). Concentrations are provided as mol% of added dye-labeled molecules relative to lipids. The typical amount of added uPAR (1.3×10^{-11} mol), as used in uPAR sequestration and dimerization experiments, is shown as solid square. Each data point is based on 20 individual readings from two bilayer samples. T-test analysis (based on two data sets with 10 data points per set) confirmed statistical significance ($p<0.01$).

Methodology: Analysis of uPAR dimerization levels

PCH curves are influenced by all of the following factors: (1) brightness of the dye labeled antibody, which is influenced by the laser power; (2) sample-specific background caused by non-specifically bound antibodies and/or fluorescence bleed through in the detection channel; and (3) detected particle numbers of monomers and dimers, which provides information about dimerization level. Because brightness and background are slightly fluctuating from sample to sample, PCH curves of different dimerization levels may look similar (e.g., PCH curves for uPAR and uPAR+vitronectin in Fig. 2). Therefore, it is very important to determine brightness and background values in separate control experiments prior to PCH analysis of receptor dimerization. In the following, the methodology is described in more detail. Specifically, to identify brightness, before and after each experiment of uPAR (with and without VN or uPA), the laser intensity was determined using a 50nM Rodamine-6-G standard solution. Next the brightness of dye-labeled antibodies in PBS solution (concentration: 1×10^{-3} mg/ml) was identified using FCS autocorrelation analysis. As verified previously using quantum dots in solution and bound to lipids in a planar lipid bilayer, the brightness of fluorescent probes in solution and associated with planar lipid bilayer is comparable in our confocal detection system (reference 29 of manuscript). For background determination, dye-labeled antibodies were added to uPAR-free bilayer samples and the fluorescence signal was determined from the bilayer following extensive rinsing. These control bilayers were produced and incubated under the exact same condition as regular experimental samples. Typically, at least one separate background sample was analyzed on each experimental day of uPAR sample analysis. With the brightness and background values being determined experimentally, the numbers of monomers and dimers are the only variable parameters in the PCH model fit analysis. The impact of variations in dimerization level (% dimer) on the shape of PCH curves at specific constant brightness and background values in our model system is illustrated in Fig. S3.

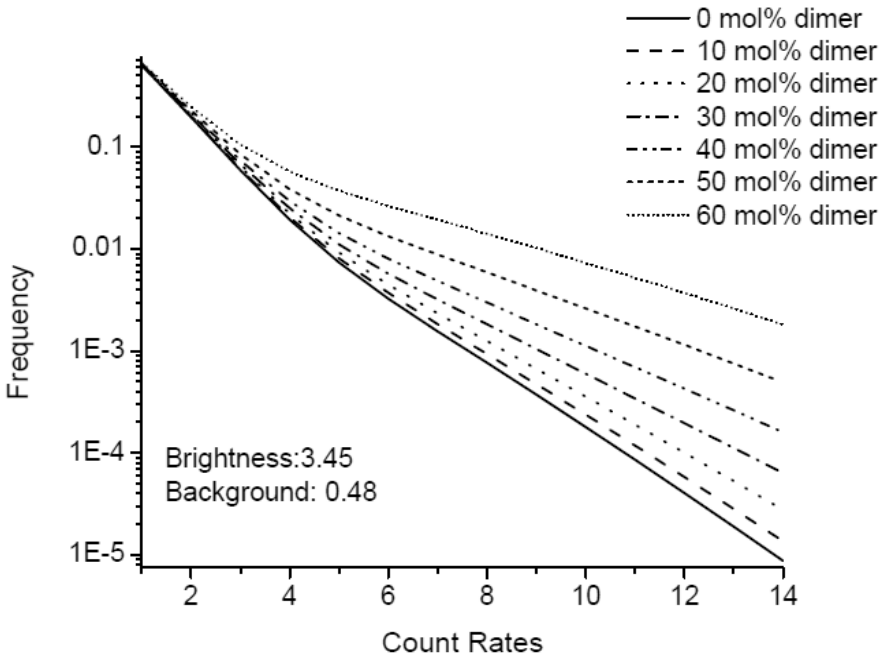

Fig. S3: PCH model curves illustrating the influence of changing dimerization levels on shapes of PCH curves (brightness and background kept constant).

FCS autocorrelation analysis of uPAR and uPAR + ligands in the bilayer

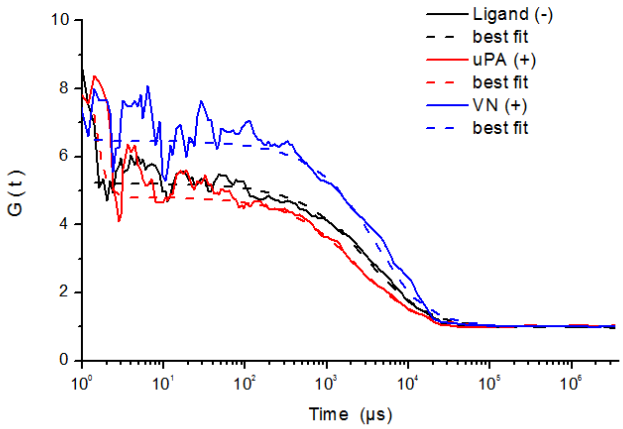

Fig. S4: Representative FCS auto-correlation data and fitting curves of uPAR, uPAR + uPA, and uPAR + VN, as obtained from FCS experiments on polymer-supported lipid bilayers. Detection of uPAR was accomplished using Alexa 555-tagged anti-uPAR MAbs.

PCH data of uPAR in binary DOPC/CHOL and ternary DOPC/DPPC/CHOL lipid mixtures

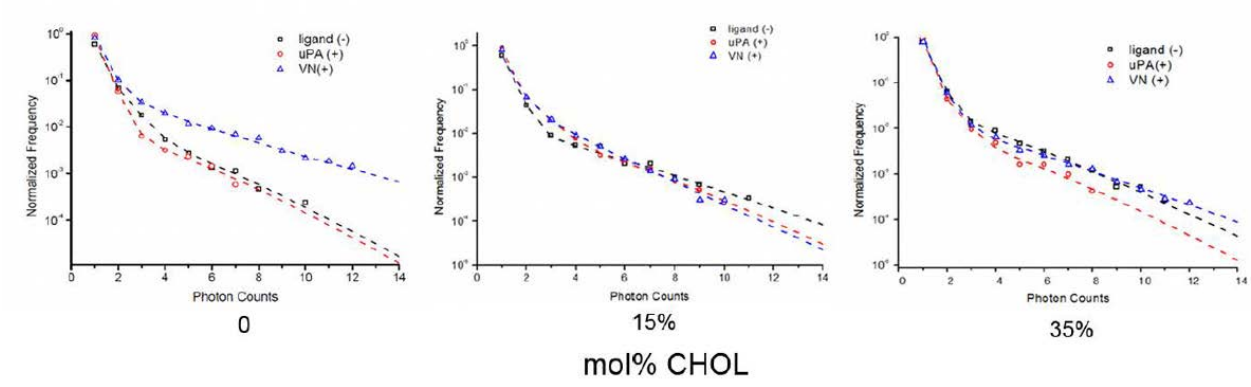

Fig. S5: Representative PCH data and fitting curves of uPAR embedded in a polymer-tethered lipid bilayer containing binary DOPC-CHOL lipid mixtures of 0 (left), 15 (center), and 35mol% (right). In each case, uPAR results are shown for three different situations: (1) uPAR without ligands, (2) uPAR + uPA, and uPAR + VN.

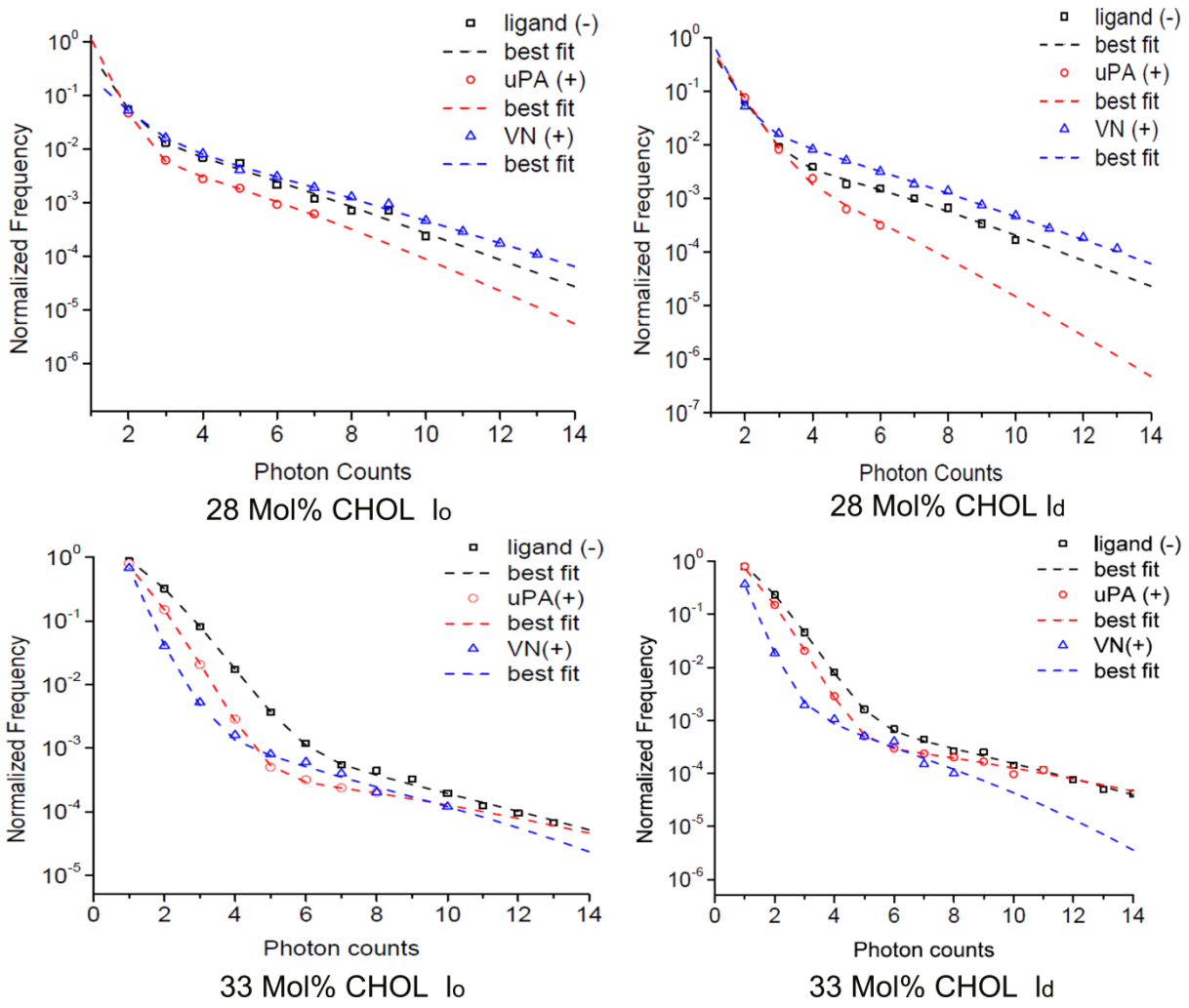

Fig. S6: Representative PCH data and fitting curves of uPAR incorporated into a polymer-tethered lipid bilayer containing ternary lipid mixtures of DOPC/DPPC/CHOL with 28mol% CHOL(top) and 33mol% CHOL (bottom) (both mixtures contain equimolar amounts of DOPC and DPPC). These raft-mimicking lipid mixtures are characterized by co-existing l_o and l_d domains. PCH data and model fits are shown separately for the l_o (left) and l_d phase regions (right). In each case, uPAR results are presented for the following three situations: (1) uPAR without ligands, (2) uPAR + uPA, and uPAR + VN.