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Amphipol mediated surface immobilization of FhuA: a platform for label-free detection of the bacteriophage protein pb5

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1. Synthesis of the B-PCApol

Chemicals were purchased from Sigma-Aldrich Chemicals Co. and used as received unless otherwise stated. Azobisisobutyronitrile (AIBN, 98%) and *N*-isopropylacrylamide (NIPAM, Acros Organic) were recrystallized twice from methanol and an acetone/hexane (4:6 v/v) mixture, respectively, prior to use. The chain transfer agent, S-1-isobutyl-S'-(α,α´-dimethyl-α´´-*N*-(isopropylacetamide)-trithiocarbonate[1], n-octylacrylamide[2] and *N*-*t*-BOC-*N*-(ethylenedioxybis(ethyl)acrylamide, phosphorylcholine glyceraldehyde[3] were synthesized as described earlier. *N*-iodoacetyl-*N*-biotinylhexylenediamine (Thermo Sci., USA) was used as obtained. 1,4-Dioxane, tetrahydrofuran (THF) and *N*,*N*-dimethylformamide (DMF) were purified by a solvent purification system with two packed columns of activated alumina provided by Innovative Technology Inc. All other solvents were of reagent grade and used as received. Thin-layer chromatography (TLC) was performed on silica gel plates (Merck Kieselgel $60F_{254}$), and preparative column chromatography was performed on Merck Si-60 silica gel (40-63 μm). Water was deionized with a Millipore Milli-Q system. Cellulose dialysis membranes (M_w cut-off 3.5×10^3 and $6\text{-}8 \times 10^3$ $g.mol^{-1}$) were obtained from Spectra/Por.

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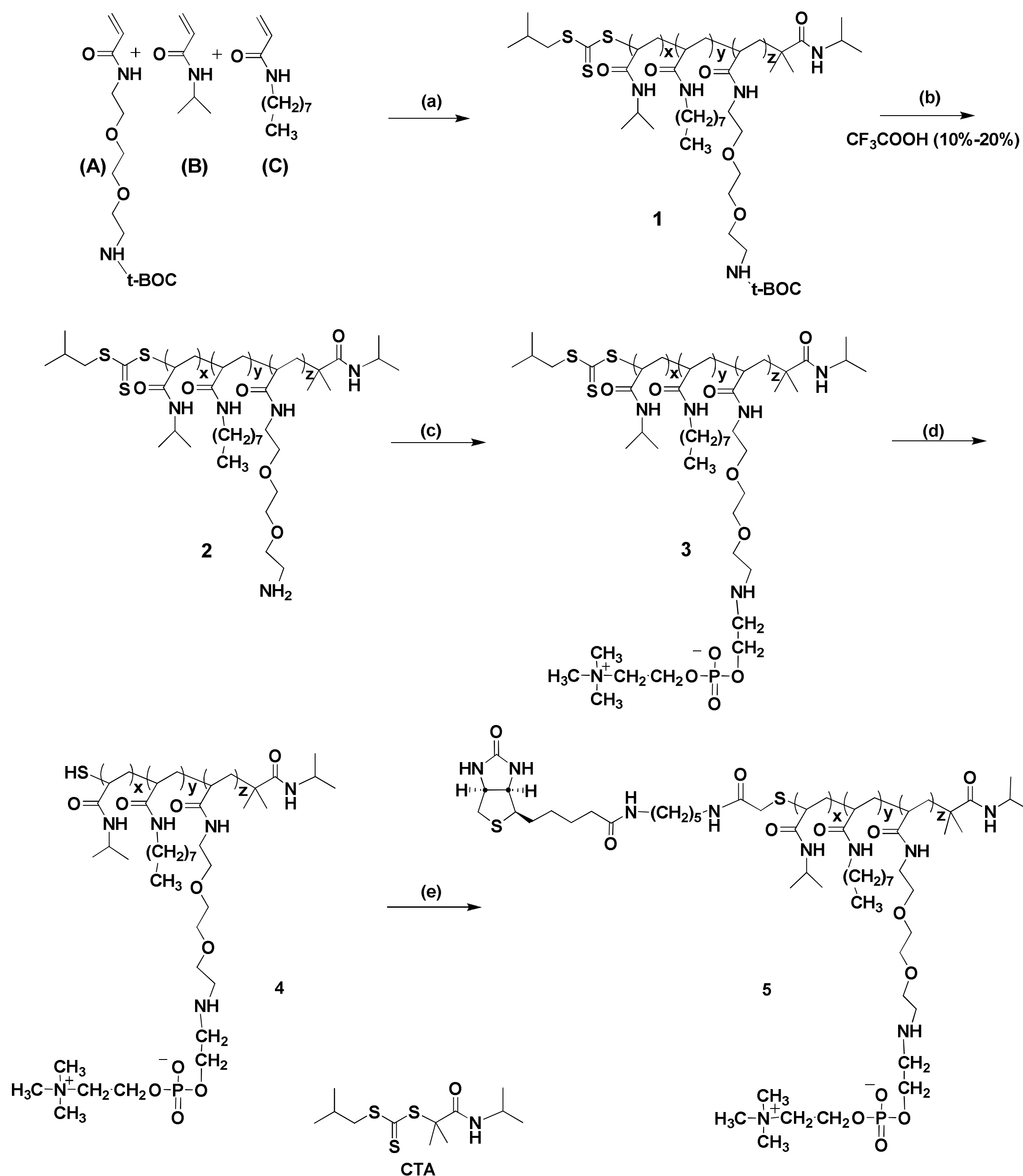

Scheme S1. Demonstrating the steps involved in the synthesis of B-PCApol

Step (a)[4] A solution of *N*-t-BOC-*N*-(ethylenedioxybis(ethyl)-acrylamide (**A**) (0.777 g, 2.57 mmol), NIPAM (**B**) (0.145 g, 1.28 mmol), n-octylacrylamide (**C**) (0.236 g, 1.28 mmol), and S-1-isobutyl-S'-(α,α′-dimethyl-α′′-N-(isopropyl-acetamide)trithiocarbonate (CTA) (22.7 mg, 0.076 mmol) in dioxane (10 mL) was degassed for 30 minutes by vigorously bubbling with nitrogen. The mixture was heated to 65°C and AIBN (5 mg) was added at once. The polymerization mixture was stirred at 65°C for 24 h, while monitoring the disappearance of the monomers by ^{1}H NMR spectroscopy. After

completion of the reaction the solvent was removed by evaporation. The residue was purified by two consecutive precipitations from THF into hexane. The polymer was dried in vacuo to yield a white powder (**1**) (0.8 g). **^{1}H NMR** ($CDCl_3$, 400 MHz, δ ppm): 0.88 (3H, -C_7H_{14}**CH_3**), 1.13 (6H, -CH(C**H_3**)$_2$, 1.26 (12H, -$NHCH_2$(C**H_2**)$_6CH_3$, 1.44 (9H, -OC(C**H_3**)$_3$, 2.86 (2H, BOC-NHC**H_2**), 3.62 (8H, CH_2 of ethylenedioxy group), 4.0 (1H, $(CH_3)_2$C**H**NHCO-), 5.4 (1H, -N**H**-); Mn: 14,300 g mol^{-1}, Mw/Mn: 1.23 (from GPC)

Step (b).[4] Trifluoroacetic acid (3.0 mL, 26.5 mmol) was added to a solution of polymer (**1**) (0.7 g) in dichloromethane (20 mL) kept in an ice/water bath. The reaction mixture was stirred for 24 h. During this time, several aliquots of methanol were added to keep the reaction mixture homogeneous. After this period, the solvent was evaporated. The polymer was purified by two precipitations from THF into diethyl ether/hexane (1:1 v/v) and dried in vacuo. The trifluoroacetate anion was replaced by the chloride anion via a 2 hour treatment of a solution of the polymer in water/ethanol (100 mL, 2:1 v/v) with Dowex 1 x 8 200-400 resins (5 g). The resin was separated by filtration, and the filtrate was concentrated in vacuo. The polymer (**2**) was isolated by lyophilization (0.52 g) **^{1}H NMR** ($CDCl_3$, 400 MHz, δ ppm): 0.92 (3H, -C_7H_{14}**CH_3**), 1.16 (6H, -CH(CH_3)$_2$, 1.33 (12H, -$NHCH_2$(C**H_2**)$_6CH_3$, 3.23 (2H, BOC-NHC**H_2**), 3.73 (8H, CH_2 of ethylenedioxy group), 3.78 (1H, $(CH_3)_2$C**H**NHCO-, 8.0 (1H, -N**H**-).

Step (c). A solution of phosphorylcholine glyceraldehydes (2.5 equiv/NH_2 equiv) in methanol (10 mL) was added to a solution of polymer (**2**), (0.2 g) in methanol (10 mL). The mixture was stirred at room temperature for 4 h. A solution of sodium cyanoborohydride (1 equiv/phosphorylcholine glyceraldehyde) in methanol (5 mL) was added dropwise to the reaction mixture kept at 0 °C. At the end of the addition the reaction mixture was warmed up to room temperature and stirred for 24 h. The polymer obtained, was purified by dialysis against deionized water for 2 days. It was isolated by lyophilization (0.22 g). The successful outcome of the conversion was ascertained from the ^{1}H NMR spectrum of the polymer (**3**), which presented a strong signal at 3.24 ppm assigned to the resonance of the phosphorylcholine methyl groups (-$^+$N(C**H_3**)$_3$).

Step (d). Polymer (**3**) (0.2 g) was dissolved in distilled and dry THF (10 mL). The solution was degassed by nitrogen for 15 min. A small amount of the reducing agent, tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was added, followed by *n*-butylamine (0.55 g, 10 fold excess compared to the thiocarbonylthio moieties). The resulting solution was stirred for 3 h at room temperature under N_2 atmosphere. The product was recovered by precipitation in diethyl ether and purified twice by further precipitations from THF into diethyl ether yielding polymer (**4**) as a white powder (0.18 g). It was used immediately to avoid possible dimerization via disulfide bond formation.

Step (e). Polymer (**4**) (0.18 mg) and *N*-iodoacetyl-*N*-biotinylhexylenediamine (24.5 mg) were dissolved in DMF (10 mL). The pH was adjusted to 7.5 by addition of an aqueous Na_2CO_3 solution. The reaction mixture was stirred for 12 h at room temperature under N_2. The resulting mixture was dialyzed for 24 h against water (cellulose membrane MWCO: 3500). Polymer (**5**) was isolated by lyophilization (0.15 mg). **^{1}H NMR** (δ, ppm, 400 MHz, $CDCl_3$): 0.916 (br s, 3H, -C_7H_{14}**C**H_3), 1.19 (br s, 6H -NHCH(**CH$_3$**)$_2$), 1.36 (s, 12H, -$NHCH_2$(C**H**$_2$)$_6CH_3$), 1.25-2.50 (m, polymer backbone), 3.24 (9H, br t, -N(C**H**$_3$)$_3$ of phosphorylcholine), 3.3-3.5 (10H, br s, CO-(C**H**$_2$)$_5$-NH- of biotin), 3.70 (br s, 8H, CH_2 of ethylenedioxy group), 3.8 (s, 1H, $(CH_3)_2$C**H**NHCO-), 4.6 (br s, 4H, C**H**$_2$ of phosphorylcholine), 4.65- 4.70 (br m, 2H, m, biotin CO-NH-C**H**). FTIR (ν, cm^{-1}): 1243 (ν PO^{2-}), 972 (ν N-CH_3).

2. Instruments and methods

2.1 NMR and FTIR: NMR spectra were recorded on a Bruker AMX-400 spectrometer operating at 400 MHz. Molar mass and polydispersity (M_w/M_n) of the polymer were determined using a GPC-MALLS system consisting of an Agilent 1100 isocratic pump, a set of TSK-gel α-M (particle size 13 µm, exclusion limit 1×10^7 g mol^{-1} for polystyrene in DMF) and a TSK-gel α-3000 (particle size 7 µm, exclusion limit 1×10^5 g mol^{-1} for polystyrene in DMF) (Tosoh Biosep) columns, a Dawn EOS multiangle laser light scattering detector λ = 690 nm (Wyatt Technology Co.), and an Optilab DSP interferometric refractometer λ = 690 nm (Wyatt Technology Co.), injection volume: 100 µL; flow rate: 0.5 mL min−1; eluent: DMF; temperature: 40 °C. Data were analyzed by the Astra 5.3.4.14 software provided by Wyatt Technology Co. UV−vis spectra were recorded on an Agilent 8452A photodiode array spectrometer. FTIR spectra were obtained on a Perkin-Elmer Spectrum One spectrometer using dispersions of polymer powder in KBr pellets. A total of 256 scans were signal-averaged in the range from 4000 to 450 cm^{-1} at a resolution of 4 cm^{-1}.

2.2 Dynamic light scattering (DLS): DLS measurements were carried out on a CGS-3 goniometer (ALV GmbH) equipped with an ALV/LSE-5003 multiple-τ digital correlator (ALV GmbH), a He−Ne laser (λ = 633 nm), and a C25P circulating water bath (Thermo Haake). The correlation functions from DLS were analyzed by the CONTIN method giving information on the distribution of decay rates (*Γ*). The apparent diffusion coefficients were obtained as $D_{app} = \Gamma/q2$, where *Γ* is the reciprocal of the characteristic decay time, $q = (\frac{4\pi n_o}{\lambda}) sin\theta / 2$ is the scattering vector, n_0 is the refractive index of the medium, λ is the wavelength of the light, and θ is the scattering angle (90°). The hydrodynamic radius (R_H) was determined using the Stokes-Einstein equation (1):

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$$D = \frac{k_B T}{6\pi \eta_s R_H} \quad (1)$$

Where, ηs is the viscosity of the solvent, k_B is the Boltzmann constant, and T is the absolute temperature. Solutions for DLS analysis were prepared by diluting the polymer stock solution (2.5 g L^{-1}) to 1 gL^{-1} in HEPES buffer. The solution was filtered through a Millex® Millipore syringe filter unit, (disposable, PVDF pore size: 0.45 µm, filter diameter: 13 mm) directly into the sample cell just before the measurement. The variation from one measurement to the next was < 5 %. The average of three measurements of 30 sec each was calculated (See Fig. S1).

2.3 SPR measurements and streptavidin functionalization: All experiments were performed on a BIAcore T100 device (Biacore AB, Sweden) operated by the BIAcore T100 Control Software 2.0.1. Measurements were performed on a CM5 sensor chip, of which the carboxymethyl dextran groups were activated as the *N*-hydroxysuccinimide (NHS) esters by a 10 min injection of a 1:1 mixture of freshly prepared 50 mM *N*-hydroxysuccinimide and 200 mM *N*-ethyl-*N*'-(dimethylaminopropyl)-carbodiimide) at a flow rate of 5 µL/min. The activated NHS ester groups were then functionalized with Streptavidin (SA) to a final value of 7000 RU, using a 1 mg/mL SA solution prepared in acetate buffer 10 mM, pH 4.5 at a flow rate of 5 µL/ min. The remaining NHS esters were then blocked by performing a 7 min injection of 1.0 M ethanolamine hydrochloride at pH 8.5 at a flow rate of 10 µL/ min. All experiments were carried out at 25 °C.

2.4 Electrochemical Impedance Spectroscopy (EIS): EIS measurements were conducted using an Autolab potentiostat 100 (Eco Chemie Utretcht Netherlands). All experiments were done on gold surfaces which were pre-functionalized to mixed self-assembled monolayers as described below; the geometric area of the surface was calculated to be 0.07 cm^2. All measurements were carried out in the 0.1 M PBS buffer as the electrolyte containing hydroquinone (10^{-3} mol L^{-1}) as the neutral redox probe. The impedance spectra were recorded between 0.1 Hz and 50 kHz at 0.12 V bias potential with ac modulation amplitude of 5 mV. A home built Teflon cell was used for the measurements. A three electrode system was used, with the substrate as the working electrode, a platinum wire as a counter electrode and Ag|AgCl|KCl (sat.) as reference electrode. The data were fitted using the ZView software (Scribner Associates Inc.) by utilizing the electrical circuit model described in the main text of the manuscript.

3. Experimental Protocols

3.1 Buffers: The preparation of the stock solution of the B-PCApol, DLS measurements, trapping of FhuA by B-PCApol and the immobilization of the FhuA-B-PCApol complex on the sensor surfaces were done in 10 mM HEPES buffer containing 150 mM NaCl and 1 mM $MgCl_2$ at pH7.4, whereas all experiments dealing with pb5 were carried out in 0.1 M PBS buffer containing 150 mM NaCl at pH 6.0.

3.2 Preparation of a stock solution of B-PCApol: A 2.5 g/L stock solution of B-PCApol was prepared by direct dissolution of the polymer (5.0 mg) in HEPES buffer (2.0 mL) followed by sonication for 5 min and gentle stirring at room temperature for at least 12 h. This solution was then filtered through a Millex® Millipore syringe filter unit (disposable, PVDF pore size: 0.22 µm, filter diameter: 13 mm) and stored in the refrigerator for not more than 2 days.

3.3 Purification of FhuA and Trapping of FhuA by B-PCApol: FhuA was extracted and purified in micelles of LDAO (*N,N*-Dimethyldodecylamine-*N*-oxide) following the protocol described elsewhere[5], except that the outer membranes were solubilized using 1% LDAO, and the Ni-NTA and ion exchange columns were performed in 0.1 and 0.05% LDAO respectively.

For the trapping of FhuA, in a typical experiment, a known volume of the B-PCApol (from the stock solution) was added to the LDAO solution of FhuA, while taking care to maintain the concentration of LDAO at 2mM (above its cmc).

In all the experiments, the final concentration of FhuA was maintained at 2.2 µM. This mixture was gently agitated and allowed to incubate for 15 minutes at room temperature. After this period, pre-equilibrated Biobeads[6] (20 g wet weight: 1g LDAO) were added to this mixture and allowed to agitate for 3 hours at 4 °C, this was followed by the renewal of Biobeads and dilution of the mixture to twice its volume by HEPES buffer and overnight agitation at 4 °C.

For determination of the optimum ratio of FhuA: B-PCApol, various mixtures ratios ranging from 1:1 up to 1:5 wt/wt of FhuA: B-PCApol were prepared as described above. UV-visible spectrum at 280 nm was recorded on each of these samples using a Nanodrop device to determine the concentration of FhuA in solution. To determine the ratio at which maximum trapping of FhuA in the B-PCApol is achieved, the sample mixtures were centrifuged at 200,000×g for 10 min. The supernatant from each tube was collected and their UV-visible spectrum at 280 nm was measured. As control experiments, 2.2 µM of FhuA in 2 mM LDAO was measured, whereas 2.2 µM of FhuA in buffer was also measured to confirm the precipitation of FhuA in the absence of adequate concentration of LDAO (see Fig. S2).

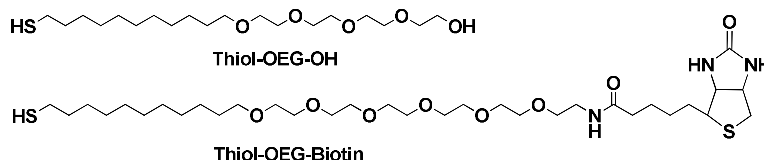
3.4 Immobilization of the FhuA: B-PCApol complex on the SA bearing SPR chip: The B-PCApol-FhuA complex was functionalized on the active flowcell of the Streptavidin functionalized CM5 chip by injecting the previously prepared FhuA: B-PCApol (1:3 wt/wt) complex at a flow rate of

2 µL/ min for a period of 30 minutes. In this complex, the concentration of FhuA was 2.2 µM and that of the B-PCApol was 0.5 g/L. The reference flowcell was functionalized with the B-PCApol alone by injecting a 0.5 g/L solution of the B-PCApol at a flow rate of 2 µL/ min for 30 minutes (Fig. S3).

3.5 Protocol for the "equilibrium analysis" measurement with pb5 by SPR: pb5 was purified according to the procedure described elsewhere.[5] Aliquots of increasing concentrations of pb5 ranging from 5 nM to 500 nM were prepared in PBS buffer at pH 6.0, from its stock solution of 5 µM concentration.

After functionalization of the SPR chip with the FhuA: B-PCApol (1:3 wt/wt) complex on the active flowcell and the B-PCApol alone on the reference flowcell, the surface was allowed to stabilize in PBS buffer, pH 6.0 for a period of 30 minutes. After this period, when the signal was found to be sufficiently stable, a few injections of the PBS buffer were performed to condition the surface. This was followed by a series of 11 injections of aliquots of increasing concentration of pb5 (of the above mentioned concentration range) at a flow rate of 20 µL/min on both the active and reference flowcells. The association or binding time of each injection was 300s whereas, in between each injection, a stabilization time of 180s was given. The reference-subtracted sensorgrams were then extracted and the data obtained were treated using a Langmuir single-site binding model using Kaleidagraph 3.5.

3.6 Preparation of mixed SAMs based surface containing the FhuA-B-PCApol complex:



Mixed SAMs were prepared from Thiol-OEG-OH and Thiol-OEG-Biotin (both procured from Prochimia surfaces and used as such) on gold surfaces. For this purpose, the gold surface was cleaned by briefly rinsing with water and drying under a stream of nitrogen; this was followed by UV-ozone treatment for 5 minutes. After this period, the surface was treated with anhydrous ethanol for 10 minutes under agitation followed by drying with nitrogen. This surface was then immersed in an ethanol solution containing 1 mM thiol-OEG-OH and 0.1 mM thiol-OEG-Biotin and left overnight. After the formation of the SAM, the surface was rinsed well with ethanol and dried using N_2.

This surface was then mounted into a home-built Teflon electrochemical cell. The SAM was allowed to hydrate in the HEPES buffer for about 15 minutes, followed by incubation with a 1 mg/mL solution of SA (in HEPES buffer) for 1 hour with intermittent agitation. The surface was then rinsed adequately with HEPES buffer and the FhuA-B-PCApol mixture (1:3 wt/wt) solution was added to the surface and allowed to incubate for 30 minutes, after which the solution was renewed in the cell and allowed to incubate for another 30 minutes. This was followed by adequate, yet gentle rinsing of

the surface with the HEPES buffer. For control experiments, the B-PCApol alone was functionalized on a SA bearing mixed SAM, exactly following the above mentioned procedure.

3.7 Protocol for Impedance measurements for the detection of pb5: For the detection of pb5 by Impedance measurements, a 10^{-3} mol/L solution of Hydroquinone was prepared in the 0.1 M PBS buffer at pH 6.0. The mixed SAM surface, bearing the FhuA-B-PCApol complex was allowed to incubate in 1 mL of this solution for 10 minutes, following which an Impedance spectrum corresponding to 0 nM of pb5 was recorded.

To this was then added a precise volume of pb5, from its 5 µM stock solution containing 10^{-3} mol/L hydroquinone in PBS buffer, in order to have a concentration of 10 nM of pb5 in the measurement cell. The surface was gently agitated and allowed to incubate for 10 min and another impedance spectrum corresponding to the 10 nM concentration of pb5 was recorded.

Similarly, further measurements corresponding to increasing concentrations of pb5 were performed by successively adding precise volumes of pb5 (from its 5 µM stock solution) into the measurement cell, and allowing it to incubate for 10 minutes before recording their data.

Duplicate measurements were performed on a fresh surface to confirm its reproducibility. Control experiment was performed by exactly following the above mentioned procedure, however, on a mixed SAM based surface bearing the B-PCApol alone to rule out the effects of non-specific interactions.

4. Supporting Figures

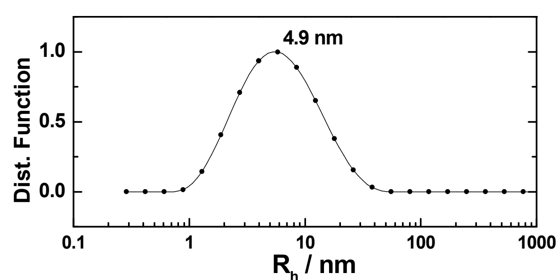


Fig. S1 DLS measurements of a 1 mg/mL solution of B-PCApol in HEPES buffer (pH 7.4).

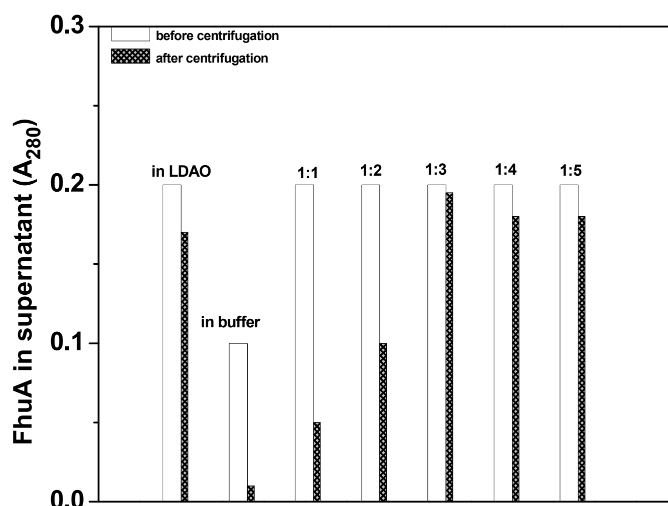


Fig.S2 UV measurements at 280 nm to determine the solubility of FhuA upon trapping with B-PCApol. The hollow bars correspond to the measurements done before centrifugation whereas the filled bars correspond to the measurements done after centrifugation. The indicated ratios correspond to FhuA: B-PCApol (wt/wt) ratios. All the samples were treated with Biobeads (3h followed by dilution, renewal of Biobeads and overnight agitation) as described above.

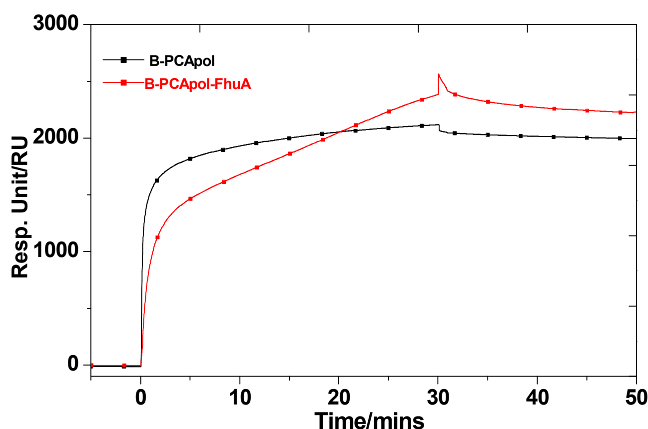


Fig. S3 Sensorgrams showing the immobilization of the B-PCApol alone (black curve) and the 1:3 FhuA: B-PCApol (wt/wt) complex (red curve) onto a Streptavidin functionalized CM5 surface. The concentration of the B-PCApol in both the samples was 0.5 g/L, whereas the concentration of FhuA in the complex was 2.2 µM.

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