ESCORTing proteins directly from whole cell-lysate for single molecule studies

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Certificate of Examination

This is to certify that the dissertation titled “ESCORTing proteins directly from whole cell-lysate for single molecule studies” submitted by Ms. Shwetha S (Reg. No. - MS11045) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Prof. Purnananda Guptasarma Dr. Arijit Kumar De Dr. Sabyasachi Rakshit

(Supervisor)

Dated: April 22, 2016

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Sabyasachi Rakshit at the Indian Institute of Science Education and Research, Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Shwetha S

April 22, 2016

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Sabyasachi Rakshit

Supervisor

April 22, 2016

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Notations

Abbreviations	Full form
ssDNA	Single stranded DNA
Cy3	cyanine 3
Cy5	cyanine 5
TIRF	Total Internal Reflection Fluorescence
AFM	Atomic Force Microscopy
SMFM	Single Molecule Force Mapping
SMFS	Single Molecule Force Spectroscopy
APTES	(3-Aminopropyl) triethoxysilane
PEG	Polyethylene glycol
RMS	Root Mean Square
CAP	Cell adhesion proteins
ESCORT	Enzymatic Sortase assisted Covalent Orientation Specific Restraint Tethering

Contents

Abstract

We have developed a method for Enzymatic Sortase-assisted Covalent Orientation-specific Restraint Tether (ESCORT)-ing of recombinant proteins onto surfaces directly from cell-lysate. With an improved surface passivation method, we obviate the cumbersome purification steps even for single molecule studies that demand high purity in the sample. We demonstrated high-specificity of the method, high-passivity of the surface and uncompromised functional integrity of anchored proteins using single molecule fluorescence and force-mapping. This method would substantially reduce the investment by way of time, money and energy in the area of single molecule studies.

Chapter 1

Introduction

Effective immobilization of proteins on substrates is key in several biotechnological applications such as chip-based detections, drug discovery and diagnostics as well as in life sciences in preparing microarrays for proteomic analysis, enzyme substrate mapping and single molecule studies. These techniques necessitate the attachment of proteins in their native, functional form on the substrate. Contemporary methods of protein immobilization on surfaces can be broadly classified into three based on the mode of interaction between the protein and surface:

- Non specific and non-covalent interaction
- Specific but non-covalent interaction
- Specific and covalent interactions

Non-specific, non-covalent immobilization of proteins on substrates suffers from several disadvantages. This method of protein attachment demands the use of highly pure proteins which often bind to the substrate in random orientations, thus reducing the efficiency of the substrate drastically. The non-selective binding of the protein also renders the method poor in reproducibility (**Figure 1.1**).

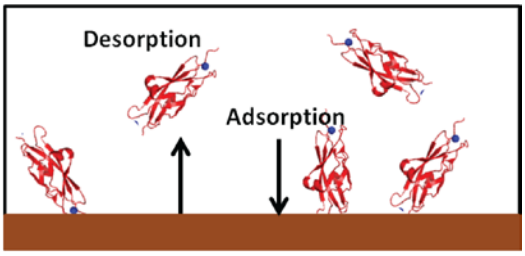

Figure 1.1 | Non-specific, non-covalent interaction between the protein and substrate suffers from several disadvantages including poor reproducibility and inadequate efficiency.

Substrates with proteins tethered via specific, non-covalent binding perform better in terms of efficiency but often the integrity of the surface is lost over subsequent assays due to the reversible nature of the non-covalent interactions. The examples of such surfaces employ the non-covalent interactions between streptavidin and biotin, nickel and nitrilotriacetic acid where one among the interacting species is appended to the protein to be immobilized and the other on the substrate (**Figure 1.2**). A major drawback of non-covalent interactions,

whether specific or non-specific, is their involvement in multiplexing measurements. Several single molecule studies investigate the strength of protein-protein interactions which are mostly non-covalent in nature. The de-attachment of the non-covalent interaction of the protein from the surface or the tip of the cantilever contributes to the lifetime of the non-covalent protein-protein interaction which is under study thus severely affecting the accuracy and precision of the study. Therefore, the most desired tethering is the specific and covalent attachment of proteins onto substrates.

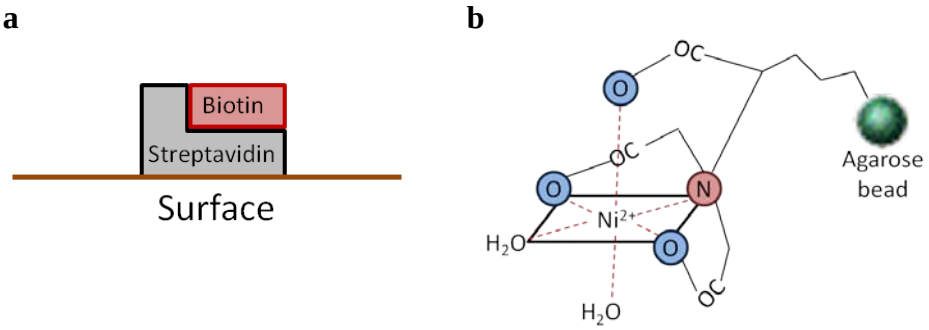

Figure 1.2 | Specific, but non-covalent interaction between the protein and substrate. (**a**) Non-covalent interaction between streptavidin and biotin. (**b**) Non-covalent interaction between Ni and nitrilotriacetic acid.

Covalent fixation of proteins on surfaces with specific orientations is crucial in single molecule force spectroscopy[1]. Covalent attachment ensures the accurate estimation of force whereas site-specific attachment steers the direction of pulling with precision. Site-specific covalent attachment of proteins with minimal nonspecificity is beneficial too in proteomic analysis on microarrays, enzyme-activity mapping, pull-down assays, affinity-based purification, wide-field single molecule fluorescence measurements, chip-based detections and diagnostics and most importantly in studying protein-protein recognition using substrate-based techniques[2].

Extant methods of covalent attachments involve large peptide tags like Halo-tag[3], Spy-tag[4] and Snap-tag[5] (**Figure 1.3**). These methods deal with complicated recombinant modifications, compromise in functional activity in proteins and suffer from multiplexing in measurements particularly in single-molecule pulling experiments[6]. HaloTag, 35kDa in size, is genetically attached to the protein of interest and covalently binds to its complimentary HaloTag ligand tethered on the substrate[3]. The large size of the tag has a strong influence on

the structural integrity and function of the protein of interest. The serious disadvantage of the use of HaloTag also includes its interference with the studied process in force spectroscopy measurements. Efforts have been made to deconvolute the contribution of Halo-tag in force spectroscopy, with an assumption of the nonsingular contribution of the peptide tag on the structural stability of the target protein[6b]. A domain belonging to the *Streptococcus pyogenes*' fibronectin-binding protein FbaB contains a Lys and Asp between which immediate isopeptide bond formation has been reported. Splitting of this domain and rational modification of the fragments is used to obtain the SpyTag which forms an amide bond to its protein partner (SpyCatcher)[4]. The limitation of this technique includes the slow reaction rate which is far from the diffusion limit. The reaction is reported to occur in minutes in μM concentration and may probably be slower or not feasible at nM concentration which is desired for single molecule studies. Also, the size of the SpyCatcher is 15kDa which might hamper the study of proteins of smaller size. The SNAP tag too suffers from the same drawbacks as it is roughly 20kDa in size. The SNAP-tag is a mutant of the human DNA repair protein O^6-alkyl guanine- DNA-alkyltransferase (^{GE}hAGT) in combination with its substrate polyethylene glycol (PEG)- benzylguanine(BG). So far, no clear indication for an event in force spectroscopy characterizing the unfolding of the ^{GE}hAGT is reported, thus making it difficult to ensure its contribution to the study of the protein under investigation[5].

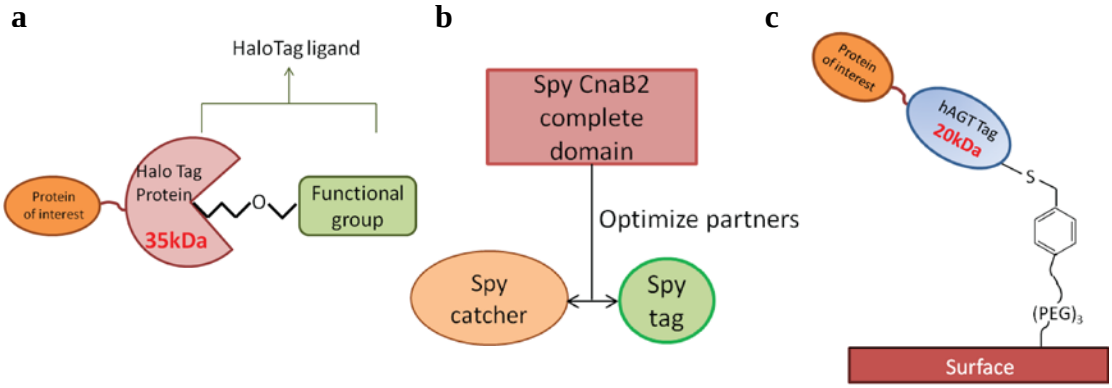

Figure 1.3 | Specific, covalent interaction between the substrate and protein is the most desirable. (**a**) Halo-Tag[3], (**b**) Spy-Tag[4], (**c**) Snap-Tag[5] are tags to covalently attach proteins on substrates but are inappropriate due to their large size and involvement in multiplexing of measurements.

Covalent attachment techniques of proteins onto the surface using cross-linkers also exist but lack specificity. Succinimidyl 6-[3-(2-(pyridyldithio)-propionamido-)hexanoate (LC-SPDP) is a long chain crosslinker for amines via its NHS ester group and cysteine sulfhydryls via its pyridyldithiol reactive groups (**Figure 1.4**). Several other cross linkers exist to tether proteins onto substrates. However, the presence of more than one lysine or cysteine residue in the protein of interest would make the cross-linking of the protein very unspecific.

Enzymatic attachments those use relatively shorter recognition sites are also restrained from universal acceptance due to the stringent buffer compositions and complicated chemical modifications (**Figure 1.5**)[7]. Protein farnesyltransferase, for example, attaches azide group to the C-terminus of proteins by recognizing short CVIA and subsequently immobilizes to surfaces exploiting the azide-alkyne chemistry. This protocol restricts the orientation of the protein to C-terminus, and more adversely, uses toxic Cu(I) for catalyzing the reaction. The Cu(I) activation of alkyne on the surface also introduces nonspecific interaction of proteins with the surface[8]. The Sfp or related phosphopantetheinyl transferase (PPTase) enzyme catalyzed immobilization attaches target protein through a Ser residue of the fused ybbR-tag to phosphopantetheine moiety of CoA attached to supporting materials. Immobilization utilizing the ybbR tag suffers from the endogenously present coenzymes in the cell lysate which often actively participate in enzymatic tethering and drastically reduce the performance efficiency and specificity[6a].

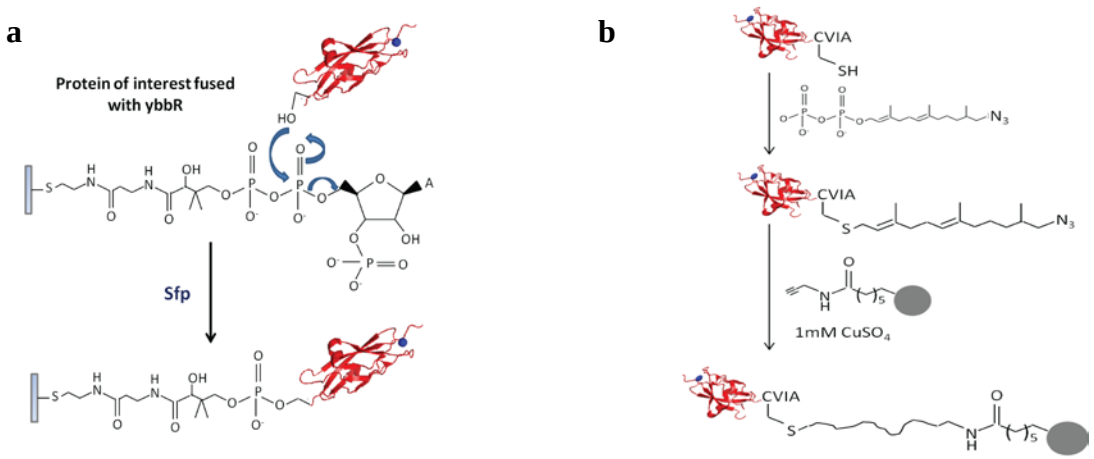

Figure 1.4 | The usage of smaller peptide tags for covalent attachment of proteins is restrained by their strict buffer compositions and complicated chemical modifications. (**a**) Covalent immobilization employing ybbR tag and phosphopantetheinyl transferase[6a]. (**b**) Covalent immobilization utilizing protein farnesyl transferase[8].

Protein purification is a demanding process in terms of time, work and investment. The cumbersome task requires standardization for efficacious reproducibility. Covalent immobilization of proteins on substrates directly from whole cell lysate is excessively advantageous. However, the method of tethering proteins should be highly specific and should involve substrates which show exceptional passiveness to the contents of the cell soup such that hardly any non-specific interactions are observed in force spectroscopy. None of the present day methods can successfully attach target proteins from whole cell-lysates with high selectivity and specificity, appropriate for single molecule experiments. Thus, the accuracy of single molecule measurements relies on the quality of the sample that necessitates multistep purification and consequently high level of protein expression.

Sortase mediated covalent immobilization of proteins has been found to be robust, ligand-specific and gentle, well suited for covalent attachment of proteins onto substrates[9]. Here, we report a method of surface passivation and subsequently **e**nzymatic **s**ortase-assisted **c**ovalent **o**rientation specific **r**estraint **t**ether (ESCORT)-ing of proteins from the whole cell-lysates appropriate for single molecule experiments. This method supersedes the cumbersome manifold purification process and efficiently attaches target proteins directly from cell-lysate with as little as nM expression.

Chapter 2

Experiment, Results and Discussion

2.1 Introduction

Exploiting the click enzymatic chemistry employing Sortase A (SrtA), we have devised a protocol for covalent, orientation specific attachment of proteins onto substrates directly from crude cell lysates. Sortase A present in *Staphylococcus Aureus*, *in vivo* catalyzes the transmembrane proteins containing enzyme-recognition motif, Leu-Pro-Xxx-Thr-Gly (LPXTG; X is any amino acid), onto the cell-wall. The cysteinyl thiol group in SrtA nucleophilically attacks the threonine-glycine amide bond and forms a proteinyl-enzyme thioester intermediate. Another nucleophilic attack by a terminal oligoglycine present on the cell-wall releases the enzyme thereby tethering the protein to the cell-wall[15]. The high propensity of the thioester towards nucleophile from the amine group of polyglycine (kinetically most preferred for tetraglycine), make sortagging extremely specific[16] and highly suitable for immobilizing proteins directly from crude cell-lysates. Non-specific interactions between the substraste and cell assemblages severely affect the efficiency of the substrate, especially for single molecule experiments. Therefore, immobilizing proteins directly from cell extracts demands highly passive surface dormant to the contents of the cell extract.

2.2 Material and methods

Functionalization of coverslips and cantilevers for passivation and subsequently covalent attachment of proteins employing sortase click chemistry.

The glass coverslips and Si_3N_4 cantilevers were first cleaned in a plasma chamber at low air pressure (typically 200- 600 mtorr) and medium radio frequency radiation (~10 MH) for 1 minute at ambient temperature. Immediately after the plasma cleaning, the surfaces were immersed in a freshly prepared piranha solution (3:1 mixture of H_2SO_4 (Merck) and 30% (w/v) H_2O_2 (Merck)) for 2 hours at room temperature. Cantilevers were placed in Piranha solution only for 30 minutes followed by gentle washing in milliQ water. Piranha solution is very corrosive and should be treated with care. The coverslips were washed under flowing milliQ water and then sonicated for 5 minutes at 53 kHz. Coverslips used in single molecule force mapping using AFM were etched in 1M KOH (Himedia) for 15 minutes. For fluorescence imaging, this step can, however, be avoided. Following the KOH treatment, the

coverslips were washed rigorously and sonicated twice in milliQ water for 5 minutes each time at 53 kHz.

The functionalization of the coverslips for covalent attachment of target proteins employing the sortagging scheme involves 3 reaction steps as following:

(i) Silanization

Silanization with aminosilane is one of the most convenient methods of turning glass, quartz or silicon based surfaces reactive. We used 3-Aminopropyltriethoxysilane (APTES, Aldrich) in 95% of acetone in water and incubated the freshly cleaned coverslips and cantilevers in it for 30 minutes. Trace of water in APTES solution facilitates the formation of the silane monolayer prior to coming in contact with surfaces and avoids inhomogeneous multilayer formation. After incubation, the surfaces were washed several times with acetone (Merck) and water and subsequently dried in nitrogen environment. The coverslip and cantilevers were then placed at 110°C for 1 hour to increase the accessibility of the reactive amine groups.

(ii) Pegylation

Polyethylene glycol (PEG), non-toxic and non-immunogenic, is known for its applications against non-specific adsorption of biomolecules or cells onto surfaces. PEG with high molecular weight also serves as spacers to surface interactions. PEG solutions were prepared with methoxy PEG-succinimidyl valerate (PEG-SVA) (Laysan Bio) and maleidmide-PEG-SVA (Laysan Bio) in PEG buffer (100mM $NaHCO_3$, 600mM H_2SO_4, pH 8.0) at a fixed concentration of 1mg/µL. Depending on the demand of the application, the distance necessitated between the single molecules might vary. 10% (v/v) of maleimide-PEG-SVA in PEG-SVA was used for majority of the experiments. To appreciate the control over the distance between the single molecules, 1% (v/v) of maleimide-PEG-SVA in PEG-SVA was used for one experiment. Following pegylation, the coverslips and cantilevers were washed with milliQ water.

(iii) Introducing polyglycine on the surface to harness proteins via the sortase mechanism

The coverslips and cantilevers were incubated in 100 µM tetraglycine to N-terminus along with cysteine at C-terminus (GGGGC) (GenScript). The cysteine is for the addition of polyglycine to the maleimide group of surface attached PEG for 7 hours at room temperature. The polyglycine stock was made in buffer A (50mM HEPES, 50mM NaCl, 25mM KCl, 2mM $CaCl_2$, pH 7.5) along with 1mM dithiothreitol (Sigma) to prevent disulphide bond formation. A large volume (50µL) of polyglycine is used for the incubation of each coverslip to sequester all the free maleimide groups on the surface to prevent its reaction with the cysteinyl group in sortase or the protein to be tethered. The coverslips and cantilevers were washed with water and stored in vacuum until their use. For their most efficient use, the coverslips must be used within 5 days for an AFM experiment and can be used until 10 days for a TIRF experiment.

Covalent, sortase assisted attachment of proteins onto the surface

200nM of purified CAP1 (and CAP2) and 240 nM of sortase were mixed such that the final volume was sufficient for the incubation of the coverslips (30µL for each coverslip) and cantilevers (30µL for each cantilever). Target protein to sortase ratio was maintained at 4:5. Buffer A was used for the reaction. Both coverslip and cantilever surfaces were incubated with the reaction mixture for 1 hour at room temperature and subsequently washed gently with the buffer to remove the unreacted CAP1 (or CAP2) and sortase.

In the case of attaching proteins directly from the cell lysate, the cell lysate was centrifuged at 13,000 rpm for 15 minutes and filtered using a 0.1µ filter. 10µL of the cell lysate was incubated with 240 nM of sortase following the same protocol as above.

Total Internal Reflection Fluorescence (TIRF) imaging

The functionality as well as the surface passivity of the ESCORT-ed coverslips were imaged using an objective based inverted TIRF microscope (Olympus model No. IX3) equipped with 488-nm, 532-nm and 633-nm diode laser systems for Alexa Fluor, Cy3 and Cy5 excitation, respectively. The fluorescence collected by an oil-immersion objective (60X, NA 1.49 Olympus) was split into two channels by a dichroic beam splitter and recorded by electron-multiplying charge-coupled device (EMCCD) camera (Q Imaging Roller Thunder). The

filters used were Quad band LF405/488/532/635-A-000 BrightLine Full Multi-Band Laser Filter set.

Proteins ESCORT-ed on coverslips were decorated with fluorophores in two different ways for imaging: immunohistochemically and enzymatically using sortase. In immunohistchemical method, the 6His-tag in the N-termini of both CAP1and CAP2 proteins were targeted. The coverslips after attaching proteins were incubated in 30µL of 1:3000 dilution of monoclonal antibody against poly-histidine produced in mouse (Sigma) for 1 hour at room temperature and subsequently washed with PBS buffer (pH 7.4), 3 times. Next, the coverslips were incubated in 30µL of 2µg/mL of 488 Alexa Fluor labeled goat anti-mouse (Invitrogen) for 1 hour at room temperature. The coverslips were again similarly washed with the same PBS buffer and imaged under the total-internal-reflection fluorescence (TIRF) microscope. Imaging was done on number of different positions to cover the entire coverslip. The same was repeated for CAP1 proteins (and CAP2 proteins) present in the cell lysate. In the enzymatic method, the C-terminus of the protein was decorated with single dye that helped in imaging of single molecule pull-down assay.

The fluorescence spots in the image were identified from the intensity maxima greater than a predetermined threshold and fitted to a two-dimensional Gaussian. The intensity threshold was kept as global constant for direct comparison between different surfaces. In case of single molecule, the single events were confirmed by single-step photobleaching. After the identification, the distance between the fluorescent spots were calculated and the shortest distances between any pair of spots was plotted.

Experiment employing TEV protease

After immobilizing proteins, the coverslip was incubated in 30µL of 1:3000 dilution of monoclonal antibody against poly-histidine produced in mouse (Sigma) for 1 hour at room temperature and subsequently washed with PBS buffer (pH 7.4), 3 times. Next, the coverslips were incubated in 30µL of 2µg/mL of 488 Alexa Fluor labeled goat anti-mouse (Invitrogen) for 1 hour at room temperature. The coverslips were again similarly washed with the same PBS buffer and imaged under TIRF. Following imaging, the coverslip was incubated on 2.5µM of TEV protease for 3 hours at 30°C. Images were taken every 30 minutes.

Test for non-specific binding and passiveness of the surface.

The passive nature of the surface was verified by fluorescence imaging using TIRF microscope and single molecule force mapping using atomic force microscope.

I. **(a)** The surface passivation against proteins were checked with 5 different cell lysates, namely, yeast cell extract, mammalian cell extract THP-1, HEK and RAW 264.7 and bacterial cell extract BL21 RIPL with Takara genes. Each of the cell lysates contained proteins of varying molecular weights.
(b) The surface passivation against non-specific DNA was checked with 40-nt ss-DNA tagged with cy3 maleimide.

The cell lysates were centrifuged at 13,000 rpm for 15 minutes. Supernatants were collected, filtered using 0.1µm filter (Millex 33mm Durapore PVDF sterile) and used directly for reaction.

Fluorescence imaging using TIRF microscope: In order to check the non-specific attachments using fluorescence signal, the reactive amine groups in the proteins in cell lysate were labeled with cy3-NHS ester (GE Healthcare) prior to incubation with surfaces. The labeled cell lysate was washed with buffer A using a 3 kDa filter until the absorbance at 545nm of the filtrate (λ_{ex} of cy3) is negligible. The absorbance of the supernatant at 545 nm was measured and the concentration of the dye attached to proteins was calculated to ensure labeling. The labeled cell lysate was incubated on the surface, in the absence of sortase, for 1 hour and gently washed with buffer prior to imaging. The same was repeated for each of the cell lysates in addition to BL21 RIPL with Takara genes. For screening the non-specific attachment of DNA (Sigma) on the surface, 50nM of cy3 maleimide (GE Healthcare) labeled 40-nt ss-DNA was incubated on the surface for 1 hour. The coverslip was gently washed and imaged using TIRF. Imaging was done in several spots so as to cover the entire coverslip. In all these experiments, the total protein concentration was maintained > 0.8 µM, much higher than required for studying protein-protein recognition of lower affinity.

Single molecule force mapping using Atomic Force Microscope (AFM): The cell lysate was incubated on the coverslip and cantilever, in the absence of sortase for 1 hour and thereafter, gently washed with buffer. Single molecule force mapping experiment was performed using an AFM on several parts of the surfaces at a constant pulling velocity of 750 $nm.s^{-1}$. The scan area for each experiment was set to 640nm * 640 nm and the step size was set according to the diameter of the cantilever tip (~10 nm, gold coated Si_3N_4 with spring constant = 30pN/nm). The stretching of the any spacer, polyethylene glycol (PEG), either from cantilever or coverslips or both was chosen as positive events and the magnitude of their unbinding forces were plotted as intensity in figures. In majority of the cases where cantilevers could not stretch any PEG, we plotted the thermal noise of the cantilever as intensity. The same was repeated for each of the cell lysate and the percentage of non-specific events was calculated for each of the cell lysates based on the positive events with respect to total.

II. Control experiments to check the extent of non-specific attachment of sortase onto the surface

The sortase was labeled with cy5 NHS (GE Healthcare) and incubated on the coverslip for 2 hours to check their adherence to modified surfaces non-specifically.

III. Control experiments to check the extent of non-specific interaction of primary and secondary antibody

30 µL of primary antibody mouse anti-his (1:3000) was incubated on the PEGylated coverslip for 1 hour at room temperature. The coverslip was then washed with PBS buffer, pH= 7.4, 3 times, 5 minute like previous cases. Next, the coverslip was incubated in 30µL of Alexa Fluor 488 labeled anti-mouse antibody from goat (2µg/mL) for 1 hour at room temperature and washed again similarly with the same PBS buffer and imaged under the TIFR microscope. Imaging was done in several parts to map the entire coverslip.

Functionality/activity of proteins post ESCORT-ed to surfaces.

Single molecule pull-down assay: Pull down of proteins from cell lysates was carried out to portray the dimerization ability, homophilically and heterophilically, of the proteins

ESCORT-ed on the surface. The pull down was monitored at the single molecule level by attaching single dye to specific counter proteins at their specific sites using sortagging. Alike ESCORT-ing, labeling target proteins with a single dye at specific site was also done in cell lysate in presence of other proteins without any purification. Pull-down mediated by heterophilic interactions were carried out by first ESCORT-ing CAP1 on the PEG modified surface as described in the protocol above. CAP2 present in the cell lysate of BL21 RIPL with Takara genes was labeled in the C-terminus employing cy3 maleimide (GE Healthcare) tagged polyglycine using the same sortagging mechanism. The cell lysate containing the C-termini labeled CAP2 was incubated on the surface already modified with CAP1 for 30 minutes. The coverslip was gently washed and imaged using TIRF to demonstrate the single molecule heterophilic pull down of CAP2.

CAP1 present in the cell lysate of BL21 RIPL with Takara genes was labeled in the C-termini following the same protocol. The coverslips were imaged under TIRF to demonstrate the single molecule homophilic pull down of CAP1.

Probing Ca^{2+} dependency on CAP-interactions to confirm high specificity in pull-down results: CAP1 was immobilized on two coverslips as per the protocol described above and subsequently imaged after labeling immunohistochemically as done before. Following imaging, one of the coverslips was incubated on buffer A containing Ca^{2+} for 2 hours as a control and the other coverslip was incubated on 10 mM EGTA for 2 hours. Both the coverslips were imaged again using same set-up.

Single molecule force spectroscopy and mapping to confirm high specificity of the surfaces: Single molecule force spectroscopy and mapping were performed for ESCORT-ed CAP1 proteins from both purified stock and from a mixture in cell lysate of BL21 RIPL with Takara genes and compared. Experiments were performed by pulling cantilevers at a constant pulling velocity of 750 $nm.s^{-1}$. The scan area for each experiment was set to 9600nm * 9600nm with the step size of 150 nm. The stretching of the spacer, polyethylene glycol (PEG), from both cantilever and coverslip were considered as positive events and compared. For mapping the force on image, the magnitude of the unbinding forces was plotted as intensity. In majority of the cases where cantilevers could not stretch any PEG, we plotted the thermal noise of the cantilever as intensity.

AFM imaging of surface and calculation of root mean square deviation.

AFM imaging was done in contact mode with cantilever (gold coated Si_3N_4 with spring constant = 10pN/nm) after the amino functionalization and pegylation of the surface. The imaging was done on multiple parts of the surface for on an area of 10µm * 10µm each.

Expression and purification of sortase.

The sortaseA (Δ59) in pET28a plasmid (Addgene plasmid # 51138) with N-terminal 6x-His tag was transformed into competent Ecoli BL21 (DE3) strain and plated onto Leuria-Bertini(LB)-kanamycin(Himedia) plates. The transformed cells were grown at 37°C in LB media till OD_{600} reached approximately 0.5 and then induced with Isopropyl- β-d-thiogalactopyranoside IPTG (Himedia) to a final concentration of 0.5mM. The cells were pelleted down after inducing the cultures at 25°C for 16hr. These pellets were processed for purification of sortaseA. 5mL of 50mM HEPES (Himedia) buffer with 100mM NaCl (Himedia), 50mM KCl (Himedia), 2mM $CaCl_2$ (Himedia) , pH 7.5 was added to re suspend the pellet after 3 freeze-thaw cycles. The bacterial cells were lysed under chilled conditions by using probe sonication working at 30% amplitude for 5 minutes with 15sec on/off pulse. The lysed cells were centrifuged at 4°C for 20 minutes and the supernatant was loaded onto pre-equilibrated Ni-NTA resin for IMAC purification. Maximum amount of protein was eluted using 200mM Imidazole (Himedia) using step elution process. The eluted protein fractions were further purified using size-exclusion chromatography with Superdex 75 16/30 column (Wipro-GE Healthcare). Purified protein was confirmed by analysing on SDS-PAGE. The protein was stored in buffer A containing 25mM arginine and 25mM glutamic acid.

Expression and purification of CAP1 and CAP2.

CAP1 and CAP2 construct was bought from GenScript and the sortase recognition site LPETG was genetically attached at the C terminus by PCR. The cell adhesion proteins were expressed in BL21 RIPL and purified using affinity chromatography followed by size exclusion chromatography with Superdex 75 16/30 column (Wipro-GE Healthcare). The purified proteins were confirmed by analysing on SDS-PAGE.

2.3 Experiment, Results and Discussion

Our surface passivation comprised two stages of necessary cleaning with plasma and fresh piranha followed by silanization and pegylation (**Figure 2.1**).

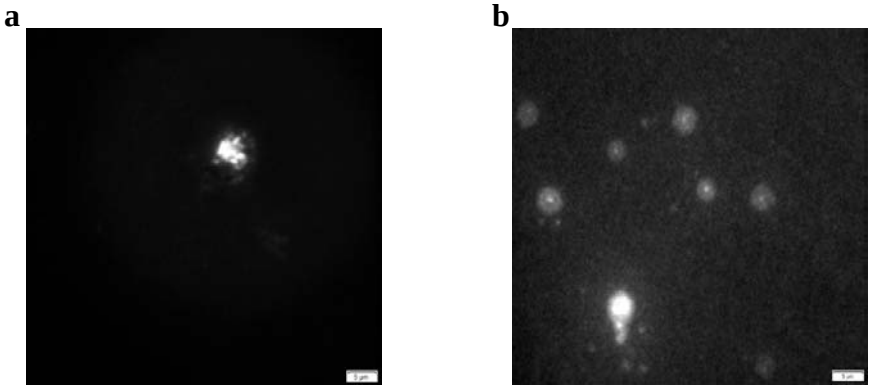

Figure 2.1 | The two stages of cleaning with plasma and piranha are necessary to avoid the predicament of scattering due to dust or charged particles on the surface. (**a** and **b**) Scattering of light due to dust particles on the surface during fluorescence experiments is extremely unfavorable for single molecule fluorescence microscopy.

We executed silanization using APTES (3-Aminopropyl) triethoxysilane) in acetone with 5% water that facilitates the formation of self-assembled monolayer by hydrolyzing the ethoxy groups in solution before impinging on the surface[10]. The RMS fluctuations in the height measured with an atomic force microscope (AFM) was 50.91 pm, reflecting a large extent of homogeneity of the APTES layer. (**Figure 2.2**)

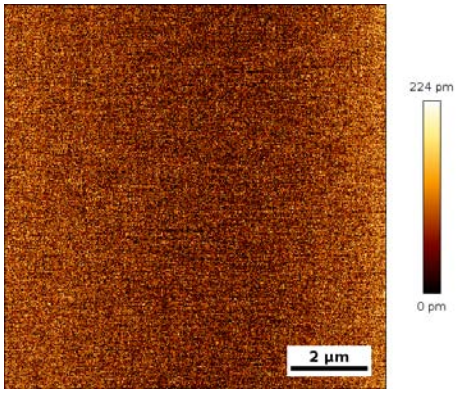

Figure 2.2 | AFM image of the surface after silanization with APTES in acetone with a trace of water. The variation in the height of the surface was measured following silanization. Minor fluctuation of **50.91 pm** confirms the homogeneous nature of the surface.

We cured the silanized surface at 110°C to steer the reactive amine groups exposed for higher accessibility[11]. Using *N*-hydroxysuccinimide(-NHS) activation to carboxylate followed by nucleophilic attack, we subsequently attached a mixture(1:10) of bi-functional (maleimide-polyethylene-glycol-NHS, mal-PEG-NHS) and mono-functional PEG-NHS to surface attached amines (**Figure 2.3**).

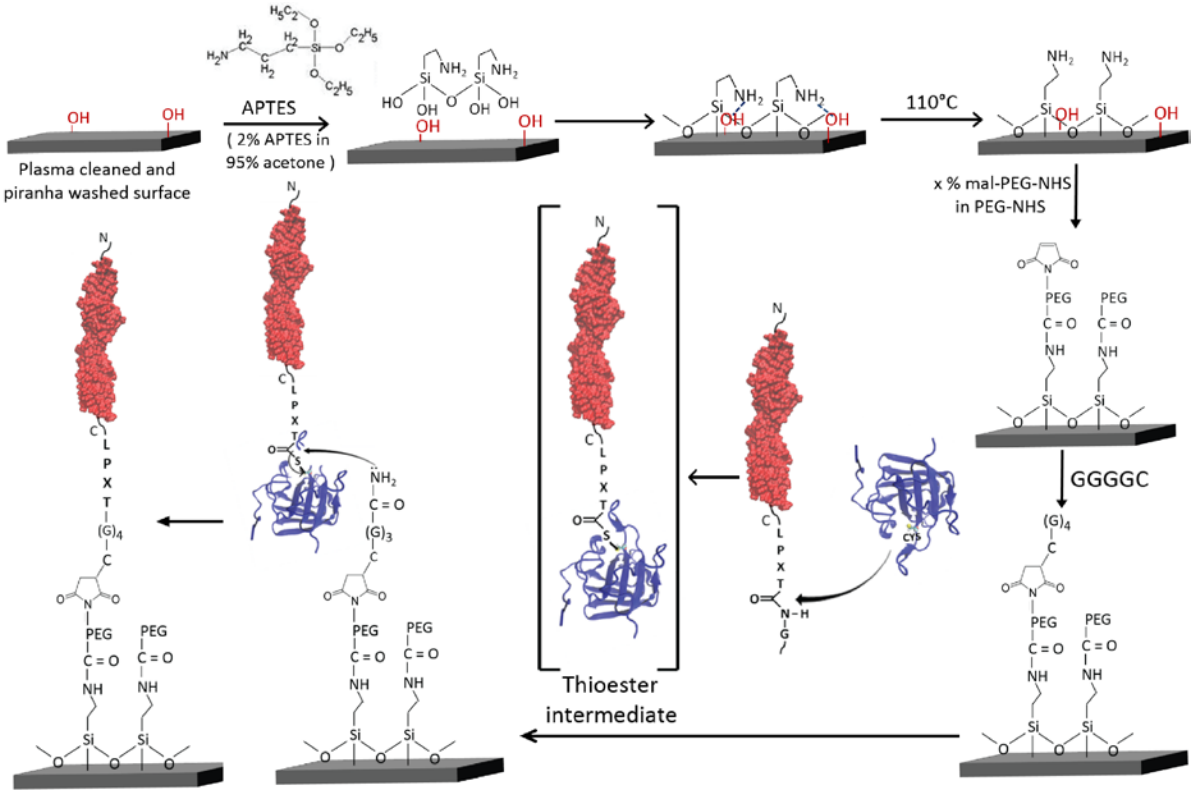

Figure 2.3 | Scheme of making highly passive surface and immobilization of recombinant proteins onto surfaces via sortagging.

PEG, non-toxic and non-immunogenic, is known to impart resistance to nonspecific adsorption of biomolecules and cells. For single-molecule force spectroscopy (SMFS), PEG as freely jointed polymer serves as a spacer[12]. We used a thermodynamically bad solvent as pegylation buffer for minimizing the surface volume and thus, maximizing the grafting density desired for surface passivation[13] (**Figure 2.4**). The bi-functional mal-PEG-NHS having mal-groups exposed to the surface was harnessed for anchoring specific target proteins.

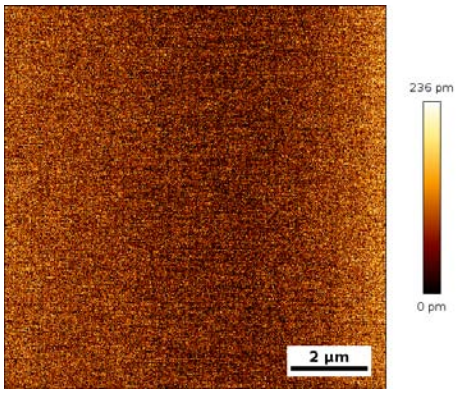

Figure 2.4 | AFM image of the surface after pegylation following silanization.
The variation in the height of the surface was measured to be equal to **53.72 pm**, affirming the homogenous nature of the surface even after pegylation.

The density of mal-PEG-NHS ensured controlled density of immobilization, critical for single-molecule studies along with chip-based detections, microarrays (**Figure 2.5**). We optimized the density of mal-PEG-NHS to 10% by monitoring the ratio of unbinding events arising from single receptor-ligand interactions to multiple using SMFS.

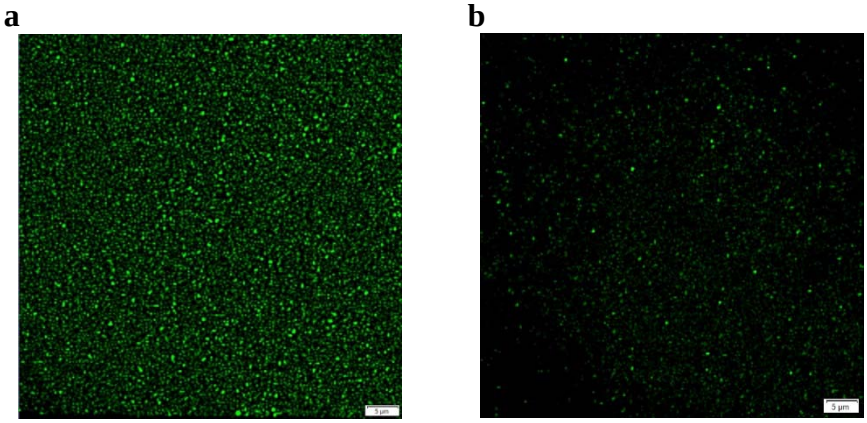

Figure 2.5 | A variation in the density of maleimide PEG can be adopted to suit different applications. (**a**) TIRF image of CAP1 ESCORTed from a chromatographically pure fraction on 10% mal-PEG-NHS modified glass coverslip. (**b**) TIRF image of chromatographically purified CAP1 ESCORTed on 1% mal-PEG-NHS modified glass coverslip.

Post-pegylation, we checked the passiveness of surface by incubating it in nonspecific proteins of wider range of molecular weights expressed in five different cell-lines varying

from bacteria to yeast and to mammalian cells using fluorescence imaging and single-molecule force-mapping (SMFM) (**Figure 2.6**). We randomly labeled the side-chain amine groups of proteins in cell-lysate with cy3 and detected no fluorescent signal w.r.t specific.

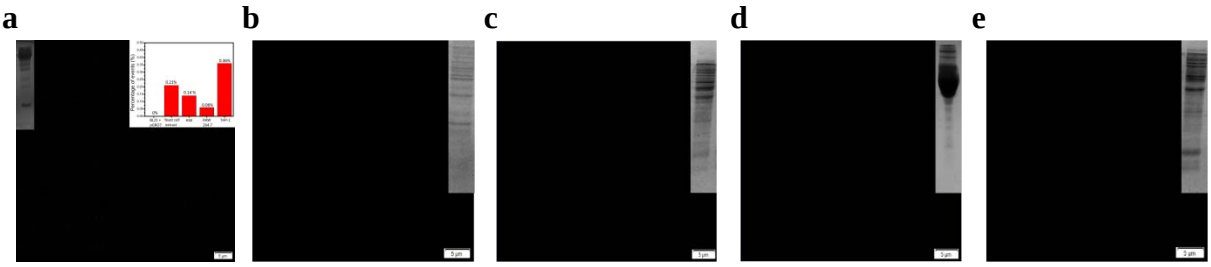

Figure 2.6 | Demonstrating the passiveness of the surfaces from the contents of yeast and mammalian cell extracts. (**a**) TIRF image of dye labeled cell lysate from BL21 RIPL in the absence of sortase. (**b**) TIRF image of dye labeled yeast cell extract in the absence of sortase (**c, d, e**) TIRF image of dye labeled mammalian cell extracts of HEK (Human Embryonic Kidney), RAW 264.7 (mouse macrophage) and THP-1 (Human leukemia monocytes) respectively, in the absence of sortase. No fluorescence signal in (**a**), (**b**), (**c**), (**d**) or (**e**) accentuates the dormant nature of the surface to the contents of the cell lysates (Inset right of (**a**, **b, c**, **d, e**): SDS-PAGE of mammalian cell extracts HEK, RAW264.7 and THP-1 respectively).

Corroborating to fluorescence results, SMFM also measured events as low as 0.5% for nonspecific incubation, nearly 20 times lower than specific (**Figure 2.7 and Figure 2.8**).

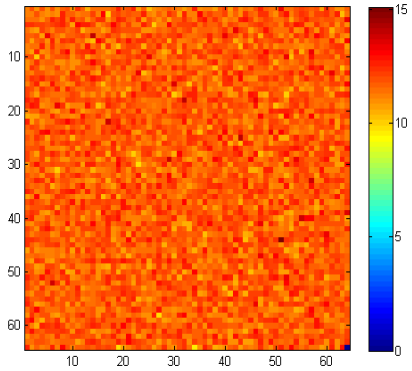

Figure 2.7 | SMFM on control surfaces where both coverslips and cantilevers were incubated with CAP1 from cell-lysate but in absence of sortase captures no events but only thermal-noise, re-illustrating the highly passive nature of the surface to the assemblages of the cell-lysate

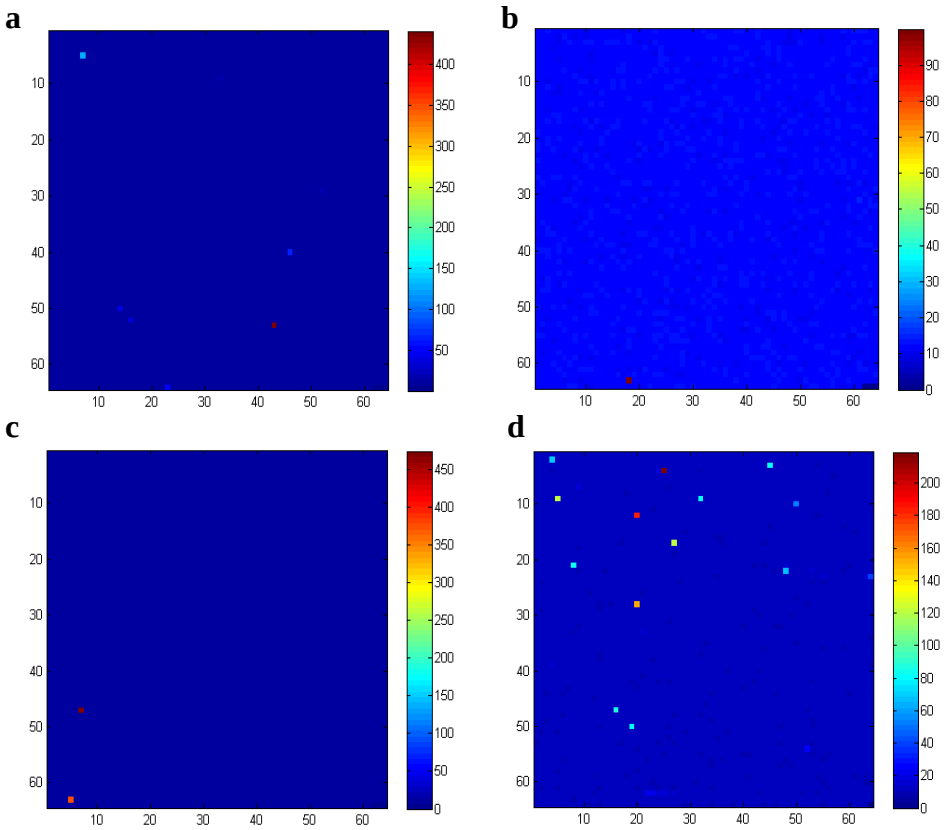

Figure 2.8 | Non-specific attachments quantified using single molecule force mapping was found to be less than 0.4%, highly appreciable for single-molecule measurements. (**a**) SMFM experiment of yeast cell extract incubated on the surface and cantilever in absence of sortase (**b**) SMFM experiment of mammalian cell extract HEK (Human Embryonic Kidney) incubated on the surface and cantilever in absence of sortase. (**c**) SMFM experiment of mammalian cell extract RAW 264.7 (mouse macrophage) incubated on the surface and cantilever in absence of sortase. (**d**) SMFM experiment of mammalian cell extract THP-1 (Human leukemia monocytes) incubated on the surface and cantilever in absence of sortase. Among all cell-lines, maximum non-specific attachment (0.4%) was observed for THP-1 cell-extract.

The higher non-specific events in force-mapping than fluorescence-imaging is probably due to the difference in labeling-efficiency of proteins as well as within detection limits of the techniques (**Table 1**).

Sl. No.	Cell lysate	Cell type	Percentage of non-specific events
1.	Bl21 RIPL + pGRO7	Bacterial	0%
2.	Yeast cell extract	Yeast	0.21%
3.	HEK (Human Embryonic Kidney)	Mammalian	0.14%
4.	RAW 264.7 (Mouse macrophage)	Mammalian	0.06%
5.	THP-1 (Human leukemia monocytes)	Mammalian	0.4%

Table 1 | The percentage of non-specific events from different cell-lines obtained from SMFM.

To screen the nonspecificity towards DNA, we incubated the surface with 40-nt ss-DNA tagged with Cy3 and noticed no detectable nonspecific attachments (**Figure 2.9**). Further passivation with protein blocking agents such as BSA did not improve the surface[14]. On the contrary, BSA facilitated nonspecific attachment from one of the mammalian cell lysates, THP-1.

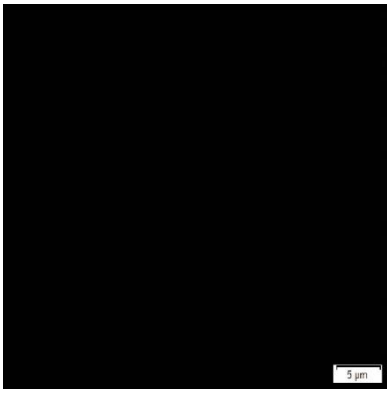

Figure 2.9 | Inertness of the surface to the non-specific attachment of DNA.
TIRF image of cyanine3 maleimide labeled DNA incubated on the surface confirms the inertness of the surface to DNA.

Using linear residue-specific enzyme reactions with bacterial transpeptidase sortase A (SrtA), we covalently attached the target proteins to the surface with specific orientations in a single step (**Figure 2.3**). Adopting sortagging, we modified two different cell-adhesion proteins (CAP1 and CAP2) with the five residues of SrtA recognition site at the C-terminus. It is well established that the short peptide chain does not interfere with any measurements and ought to be to serving as a linker to accommodate the possible rotational degree of freedom[12]. Polyglycines those are pre-attached to surface through maleimide-cysteine click chemistry, serve as a nucleophile to attack the thioester and engrafts proteins covalently onto surface (**Figure 2.3**).

To confirm the covalent bond as the only mode of association between protein and surface, we incubated CAP1 and CAP2 in polyglycine attached surfaces in absence and presence of SrtA. Immunohistochemistry against N-terminus 6his-tag led to a TIRF signal only for SrtA mediated attachments, thereby ensuring specific and only covalent interactions of CAP1 and CAP2 with the surface (**Figure 2.10**).

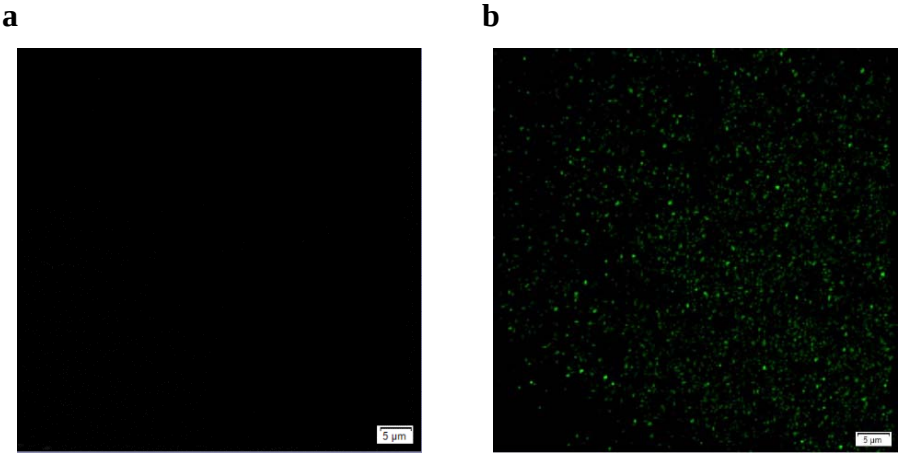

Figure 2.10 | To demonstrate the covalent bond formation as the only interaction due to sortagging (a,b), TIRF image of CAP1 was recorded in absence of sortase (**a**) and presence of sortase (**b**) on 1% mal–PEG-NHS coverslip.

To nullify the non-specific interaction of sortase with the surface, we labeled the lysines in sortase cy5 NHS ester. We incubated the labeled sortase on the surface for 2 hours and then

washed the surface. We observed the surfaces employing TIRF **(Figure 2.11)**.

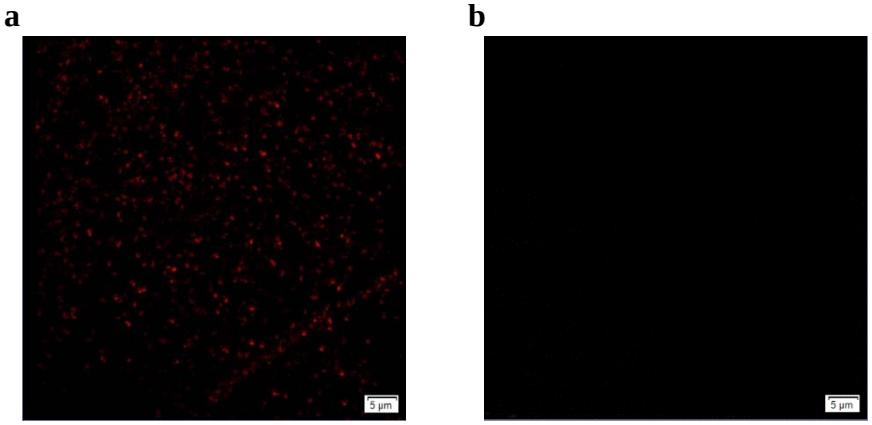

Figure 2.11 | To demonstrate that sortase does not adhere to the surface non-specifically. TIRF image of cyanine5 labeled sortase A incubated on surface for 2 hours (**a**) before washing and (**b**) after washing with buffer.

To screen the high-specificity of sortagging and effective inertness of the surface, we compared surfaces decorated by ESCORTing proteins (CAP1 or CAP2) from whole cell-lysate and from chromatography-purified fractions. We carried out immunohistochemical imaging for both surfaces using TIRF with primary and Alexa Fluor 488 labeled secondary antibody against N-terminal 6his-tag (**Figure 2.12**).

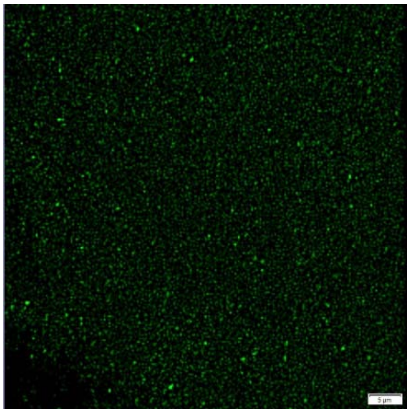

Figure 2.12 | TIRF image of CAP1 ESCORTed from a crude cell extract directly on 10% mal-PEG-NHS. Identical intensity distribution of both the images (**Figure 2.5a** and **Figure 2.12**) underscore the high-passivity of the surface and exceeding specificity of sortagging.

As expected, we observed comparable density of fluorophores along with identical distance distributions between closest fluorescent spots for both cell-lysate and purified proteins (**Figure 2.13**).

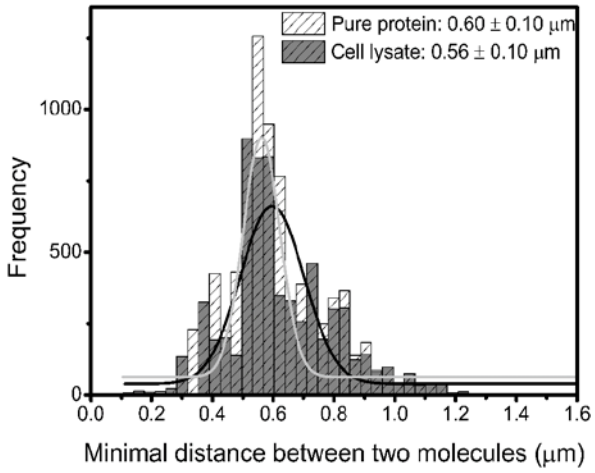

Figure 2.13 | An overlay of distance distribution of two closest fluorescent spots obtained from Figure 2.5a and Figure 2.11 portray an equivalent distribution centered at (0.56 ± 0.01) µm for crude cell extract and (0.60 ± 0.01) µm for purified protein.

To distinguish the specific fluorescent spots from nonspecific artifacts, we employed TEV protease to cut the N-terminal 6his-tag site and observed >99% reduction in fluorescent signal (**Figure 2.14**).

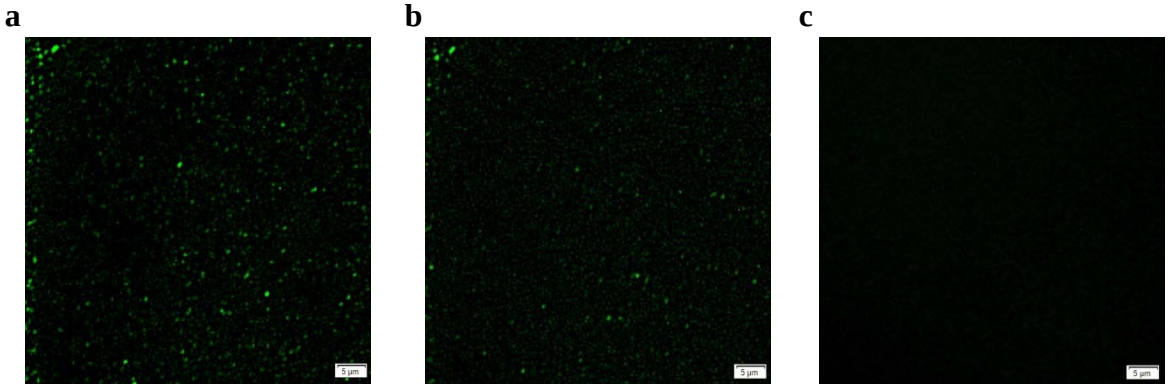

Figure 2.14 | Illustrating >99% reduction in fluorescence signal after employing TEV protease. (**a**) TIRF image of CAP1 protein immobilized on the surface. The signal is seen from the fluorescently labeled immunohistochemistry against the N-terminus 6his-tag. (**b**) A reduction of fluorescence signal is obtained following the incubation of the surface on TEV protease of 2.5µM for 30 minutes. (**c**) The fluorescent spots vanished as TEV protease cleaved almost all the 6his-tag after 3 hours of incubation of surface on TEV protease.

To confirm no non-specific interaction of the primary and secondary antibodies, we incubated the primary antibody and subsequently the secondary antibody for 1 hour each on the coverslip and washed the coverslip with buffer (**Figure 2.15**). We observed the coverslip employing TIRF.

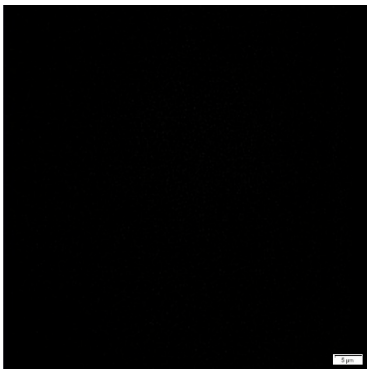

Figure 2.15 | No non-specific interaction of the primary and secondary antibody with the surface.

It is the fundamental requirement for a surface to maintain the functionality of the proteins after immobilization. We probed the Ca^{2+}-dependent protein-protein interactions of cell-adhesion proteins (CAP1 & CAP2) to check the biocompatibility of the modified surfaces. Both CAP1 and CAP2 interact homophilically as well heterophilically through their N-termini in *trans* conformation. To visualize such dimerization on surfaces, we carried out single-molecule pull-down of ligand-proteins (CAP1 or CAP2) from whole cell-lysate. We first ESCORT-ed receptor-proteins (CAP1 or CAP2) to surfaces. Ligand-proteins in cell-lysate were fused with single cy3 at their C-terminals by manifesting sortagging and allowed them to interact with their receptors ESCORTed already on surfaces (**Figure 2.16**).

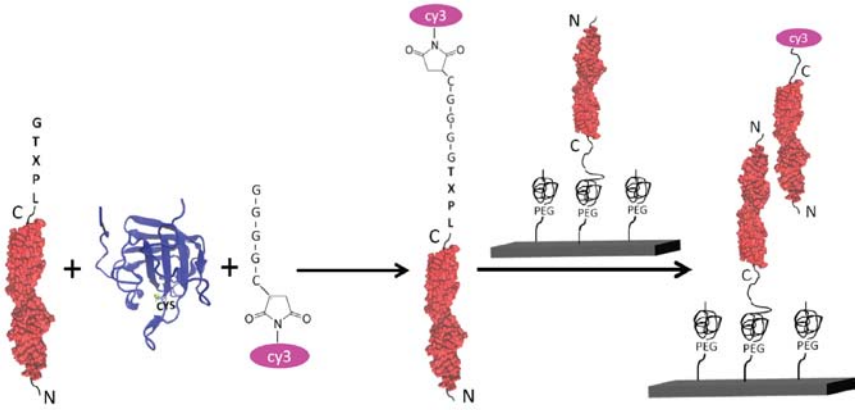

Figure 2.16 | Scheme for labeling CAP1 proteins in the cell-lysate with a single cy3 using sortaseA and subsequently performing single molecule pull-down.

We imaged the surfaces using TIRFM (**Figure 2.17a and b**). In control, the absence of ESCORTed receptor-proteins on the surface did not attract ligands and showed no TIRF signal (**Figure 2.17c**).

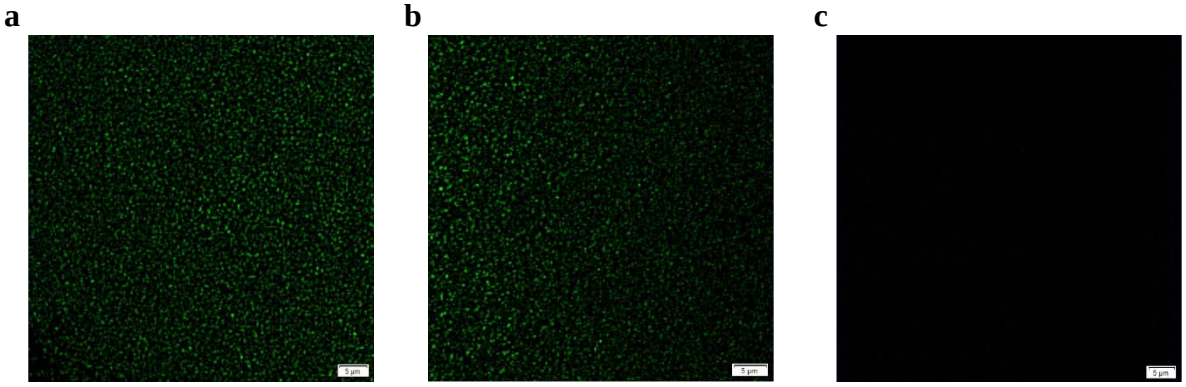

Figure 2.17 | Functionality of the ESCORTed proteins on the substrate.
(**a**) Cy3-modified CAP1 from cell-lysate was pulled down by the ESCORTed receptor CAP1 on surfaces at the single molecule resolution as imaged under TIRF microscope. (**b**) Cy3-modified CAP2 from cell-lysate was pulled down by the ESCORTed receptor CAP1 on surfaces at the single molecule resolution as imaged under TIRF microscope. (**c**) The absence of ESCORTed proteins on the surface did not attract any ligands and showed no fluorescence signal.

We measured the mean distance between the closest fluorescent spots, identified by single-step photobleaching as 840 nm. Since the ratio between the unlabeled protein to labeled one was set to 10:3 to identify single molecules, we averaged the distance obtained by 3.3. The mean distance between the proteins was measured as 252 nm ± 10 nm (**Figure 2.18**).

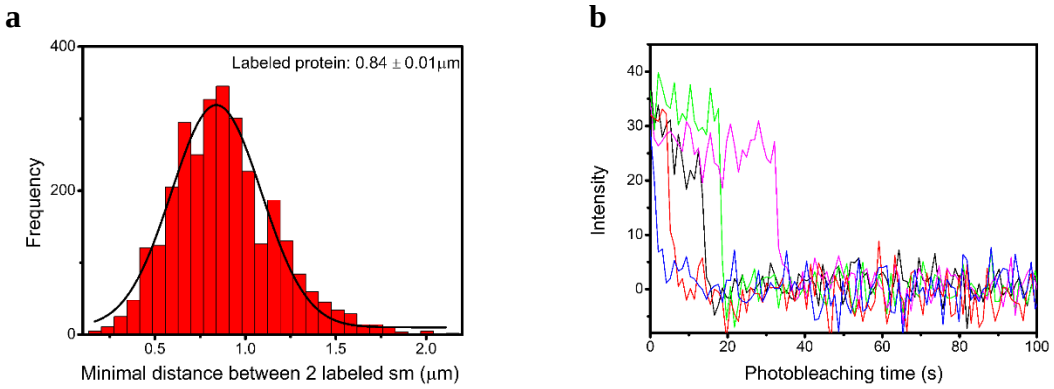

Figure 2.18 | Distance distribution of single CAP1 proteins. (**a**) The mean distance between the pull-down proteins is 252 nm for 10% mal–PEG-NHS surface. (**b**) A sharp, single drop in the fluorescence intensity in the plot between intensity and time, characteristic for single molecule was observed for almost all fluorescent spots, among which 5 have been represented above.

To check the extent and specificity of single molecule pull-down, we probed the unbinding force of protein-protein interactions at the single-molecule level using AFM. We ESCORTed purified CAP1 on the cantilever in addition to the glass-coverslip and subsequently quantified the strength of interaction at a constant pulling velocity of 750nm/s. Next, we repeated the experiments by ESCORTing CAP1 from cell-lysate on both coverslip and cantilever and measured the distribution of unbinding forces (**Figure 2.19**).

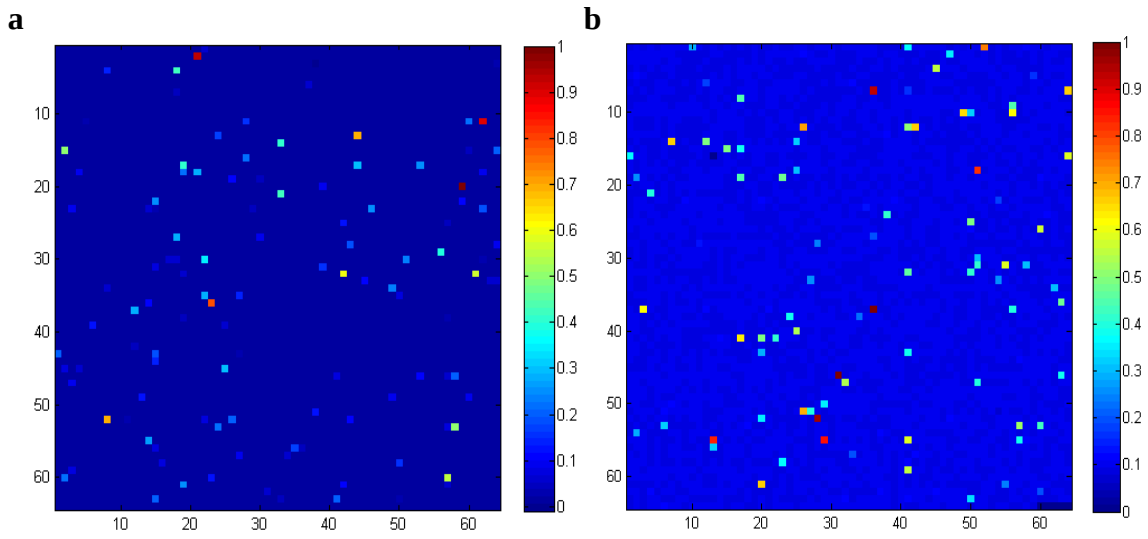

Figure 2.19 | Single molecule force mapping experiments to probe the unbinding force of the protein-protein interaction. (**a**) Single-molecule force-mapping between cantilever and surface ESCORTed with CAP1 from chromatographically pure fraction is shown in colormap by normalizing with the maximum force. (**b**) Single-molecule force-mapping between cantilever and surface ESCORTed with CAP1 from crude cell-extract is shown in colormap by normalizing with the maximum force.

We measured an identical distribution of unbinding forces for the protein-protein interaction when chromatograpically pure fraction of CAP1 was ESCORTed as compared to CAP1 directly from crude cell-extract (**Figure 2.20**). This highlights the high-passivity of the surfaces towards nonspecific attachments from cell-debris.

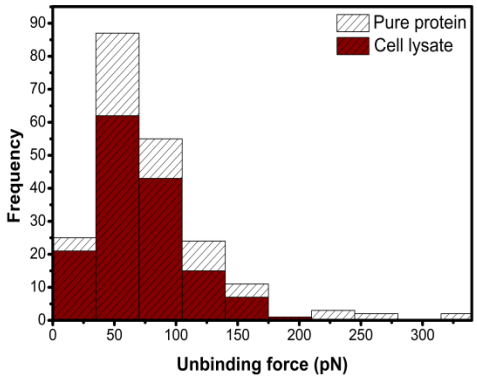

Figure 2.20 | An overlay of the distribution of unbinding forces of interactions between CAP1-CAP1, ESCORT-ed from the pure fraction and cell lysate show identical nature.

The probability of interactions with cell-lysate proteins was 1.4x lower than the pure fraction. We also observed 3.5% multiple among all events with proteins ESCORTed from cell-lysate and 1.9% for pure fraction (**Figure 2.21**).

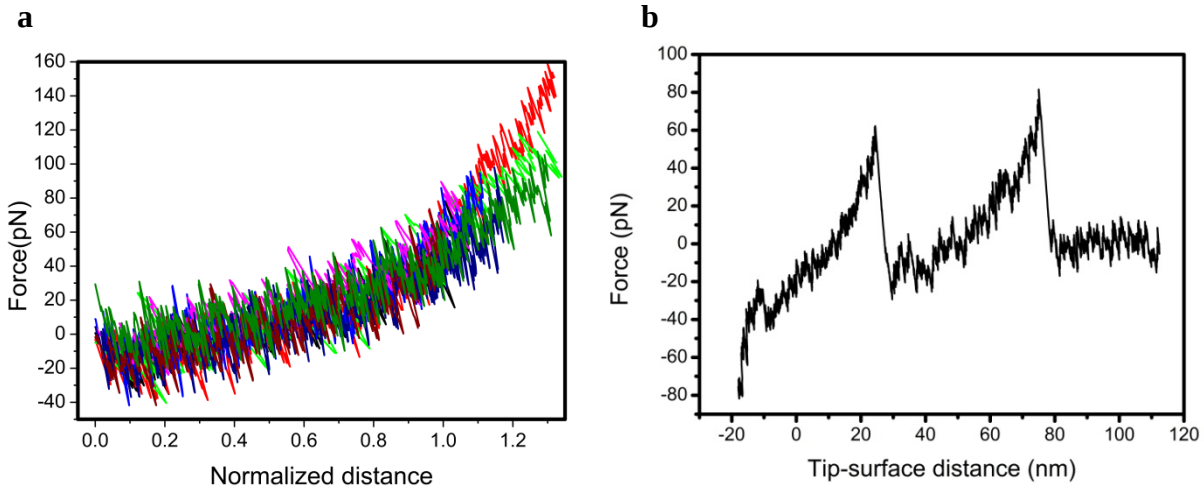

Figure 2.21 | Events observed in single molecule force mapping experiments.
(**a**) Single molecule events characterized by PEG stretch normalized by the tip-surface distance of the least binding force of the 8 force curves represented here. (**b**) Events characterized by more than one PEG stretch were considered as multiple events.

We confirmed the specificity of the interactions by probing its Ca^{2+}-dependency. (**Figure 2.22**)

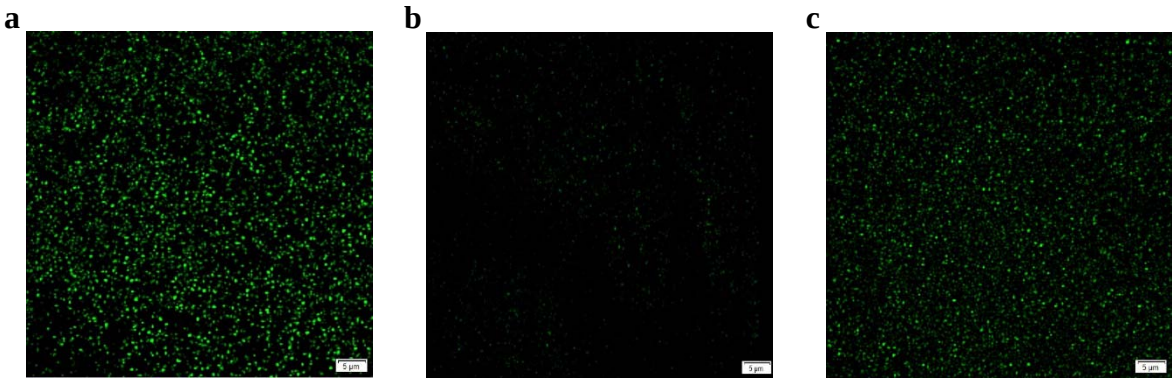

Figure 2.22 | Probing of Ca^{2+} dependent protein-protein interactions of cell-adhesion protein1 (CAP1). (**a**) TIRF image of fluorescently labeled C termini CAP1 protein interacting with the immobilized CAP1 protein. (**b**) TIRF image of the surface after incubation on 1 mM EGTA buffer for 1 hour. EGTA, a well-known chelator, complexes out the Ca^{2+} thereby preventing the interaction between CAP1 and CAP1 which is seen as a decrease in the fluorescently labeled spots. (**c**) TIRF image of the surface after incubation on 2mM Ca^{2+} buffer for 1 hour. Any decrease in intensity can be attributed to the dissociation of the fluorescently labeled immunochemistry against the 6 His-tag.

2.4 Conclusion

To conclude, we have devised a protocol for effective surface passivation and ESCORT-ing of proteins which can be suitably deployed for single molecule studies. The exceedingly high passive nature of the surface and high specificity of the sortase reaction is apt for protein ESCORT-ing directly from cell-lysate deploying the protocol substantial reduction in investment of time, energy and chemicals in purification. Sortase mediated reactions are strictly site-directed and hence, can be targeted for labeling any unstructured part in addition to C, N-terminals[15, 17]. The availability of both Ca^{2+}-dependent and independent variants of sortase make the labeling more pliable to buffer[18]. Since sortagging is also reported on DNA our protocol can be extended to ESCORT DNA[19] as also to any cellular-organelle onto surfaces directly from cell-lysate.

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