Stable and Reversible Functionalization and Super-Resolution Microscopy of Live Cell Membranes

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Materials and Methods

Materials
All the chemicals including NHS-fluorescein, AF647 NHS-ester, adenosine di-phosphate (ADP), poly-D-lysine etc. chemicals and analytical grade solvents were purchased from commercial suppliers and used without further purification. Cell viability and proliferation assays were performed using cell titer blue (CTB) reagent. Fluorescence images of live cells were acquired using 60X objective in confocal laser microscope (Nikon AR1-MP) and 3D confocal movies were generated as supporting information. Real time growth and images of live cells were monitored by IncuCyte S3 live cell analysis system.

Cell culture
Jurkat T cells were obtained from Dr. Majid Kazemian lab (Purdue University, USA) and cells were cultured following American Type Culture Collection (ATCC) protocol at 37°C with 5% CO₂ atmosphere in a humidified incubator. For normal growth, Jurkat T cells were cultured in RPMI-1640 media (Gibco) supplemented with 10% FBS (Atlanta Biologics), 20 mM HEPES and 1% penicillin/streptomycin (Invitrogen). All the cargo compounds were dissolved in PBS at high concentration (1 mg/mL) followed by filtrations using a 0.22 µm syringe filter and dilutions from this stock solution were prepared in culture medium.

NMR Sample preparation and data analysis:
10 mg of the solid cargo sample was dissolved in 0.6 ml D₂O by sonication only at RT. ¹H-NMR spectra were recorded using a 500 MHz spectrometer (Bruker AV-III-500-HD) at room temperature. Number of Scan (NS) for each experiment was 256. Chemical shifts (δ) are reported with D₂O (δ = 4.80 ppm) as internal standards. Analysis and ¹H-NMR figures were prepared using MestReNova software.
Super-resolution imaging

Super-resolution imaging was performed using a custom-built 3D super-resolution system with biplane configuration with a 100× 1.35 NA silicone oil-immersion objective lens (FV-U2B714, Olympus America Inc.) and a PIFOC objective positioner (ND72Z2LAQ, Physik Instrumente). Samples were excited with a 642 nm laser (2RU-VFL-P-2000-642-B1R, MPB Communications Inc.). Single molecule emission events were captured by a scientific complementary metal-oxide-semiconductor camera (Orca-Flash4.0v3, Hamamatsu) with an effective pixel size of 120 nm. 3D volume was formed by scanning the objective lens in the axial direction with 400 nm interval per optics section. 3D super-resolution images were reconstructed from 8 optics sections with each section containing 6,000 frames imaged through 3 cycles. Single molecule 3D position estimation was performed by a in situ point spread function retrieval method as described previously by F. Xu et al.¹

Synthesis

Synthesis of covalent cargo backbone.

Step 1. Synthesis of benzo-triazolide of iodoacetic acid

Dry thionyl chloride (1.1 mmol) was added to a solution of 1H-benzotriazole (3 mmol) in 10 ml dry DCM taken in a 50 ml RB at room temperature under Ar atmosphere. The reaction mixture was stirred (500 rpm) for 10 minutes. Next, solid iodoacetic acid (1.0 mmol) was dissolved in 10 ml of dry DCM in a closed cap vial and added quickly. The reaction mixture was stirred for 12 h at room temperature. The white precipitate was filtered off and the filtrate was concentrated under
reduced pressure. After the evaporation of the solvent, the crude residue of benzotriazolide was isolated as intermediate and used in the next step reaction. This benzotriazolide intermediate should be used immediately in the next step before it turned into blackish from pale yellow/brown color, as it results in product degradation.

**Step 2. Synthesis of cargo intermediate I**

30 mg HBr salt of Poly-D-Lysine (M ~150 kD) was dissolved in 5 mL MeOH followed by addition of 100 µL triethyl amine. This mixture was stirred for 30 minutes at 4°C. Next, the crude benzo-triazolide (0.06 mmol) obtained in the step 1 was added and the reaction mixture was stirred at 4°C for 24 h. After evaporation of the solvent MeOH at RT (inside hood), crude residue of cargo intermediate I was isolated as precipitate which was purified by washing with acetonitrile and used in the next step. This crude cargo intermediate I should not be dried completely (to solid state), otherwise some changes may happen in the polymer and it may be difficult to dissolve in next step. This product needs to be dried until semi-solid or colloidal state of the product.

**Step 3. Synthesis of cargo intermediate II**

Adenosine diphosphate (ADP) free acid (5.3 mg) was dissolved in 5 mL THF in an RB followed by addition of 2.4 mg of 4-dimethyl amino pyridine (DMAP) to it. This mixture was stirred for 1 h at 4°C followed by addition of cargo intermediate I (30 mg) dissolved in 5 ml MeOH. The resulting reaction mixture was further stirred for 24 h at 4°C. The reaction mixture was acidified with ice cold 1 (N) HCl to neutralize any remaining base. Finally, the solvent was evaporated at RT (inside hood) to get the crude product of covalent cargo which was purified by washing with acetonitrile. ADP used in this step should be in free acid form and not in salt form. Free acid form will help to dissolve in organic solvent like THF. This crude cargo intermediate II should be
washed with acetonitrile to make sure there is no small molecule like ADP, DMAP etc. Presence of these nucleophilic small molecules will compete with the amine group of the cargo back bone against the electrophilic site of the NHS-Fluorescein in the next step - leading to decrease in degree of fluorophore conjugation on the cargo back bone. Few drops of 1 (N) HCl can be used to dissolve this cargo intermediate for next step reaction if necessary.

**Step 4. Synthesis of fluorophore tag covalent cargo molecule**

6.0 mg of cargo intermediate II prepared in the previous step was dissolved in 1 ml of water followed by addition of 4 mL methanol and 100 μL of triethyl amine. This mixture was stirred for 30 minutes at 4°C followed by addition of 1.0 mg NHS-Fluorescein (dissolved in 1 ml MeOH). The resulting reaction mixture was further stirred for 12 h at 4 °C. Un-reacted triethyl amine was neutralized by dropwise addition of ice cold 1 (N) HCl. The orange-yellow precipitate obtained from this reaction was separated and washed with cold methanol and acetonitrile and dried at room temperature (inside hood) to obtain the final fluorophore tag cargo molecule. This final cargo molecule should be neutralized with 1 (N) HCl to avoid aerial oxidation of the product and can be dried to solid state. This product can be dissolved in acidic PBS for cell conjugation and other work.

**Synthesis of non-covalent phospholipid cargo**

4.0 mg of HBr salt of D-lysine chain polymer (150 kD) was dissolved in 5 mL water. 10 μL triethyl amine was added and mixture was stirred for 5 minutes at room temperature (RT) to free all amine groups. Next, 2.0 mg phospholipid was dissolved in 2 mL methanol and added to it and the reaction mixture was stirred at RT. The reaction mixture was evaporated to get the crude residue and
washed with acetonitrile to remove any un-reacted phospholipid and get the non-covalent phospholipid cargo.

**Synthesis of covalent phospholipid cargo**

6.0 mg of covalent cargo was dissolved in 5 mL water followed by addition of 2.0 mg phospholipid and the reaction mixture was stirred at room temperature. The reaction mixture was evaporated at room temperature to get the crude residue, then washed with cold acetonitrile to remove any un-reacted phospholipid and get the title product.

**Synthesis of non-covalent fluorophore cargo**

Synthesis scheme for fluorophore conjugated cargo molecules is shown in Supplementary Figure S5. For non-covalent fluorophore cargo molecule, 8.0 mg HBr salt of D-lysine chain polymer was dissolved in 5 mL methanol by adding 100 µL triethyl amine. The resulting reaction mixture for the non-covalent cargo reactions (Supplementary Figure S4) was further stirred for 12 h at 40°C. Un-reacted triethyl amine was neutralized by dropwise addition of ice cold 1 (N) HCl. The orange-yellow precipitate obtained from this reaction was separated and washed with cold methanol and dried at room temperature in vacuum to obtain the dual-conjugated fluorophore covalent cargo.

**Synthesis of covalent cargo conjugated with AF647 dye**

A solution of poly-D-lysine conjugated with ADP (dissolved in MeOH) was stirred for 30 minutes at 4°C, then 1 mg of AF647 NHS ester was added. The mixture was stirred at 4°C overnight. The mixture was purified with protein pierce concentrator with 10K MWCO by dissolving the product in 70% ethanol.
Synthesis of covalent cargo 1 (75 kD) and covalent cargo 3 (300 kD)

Both the covalent cargo 1 and 3 were synthesized as per the above covalent cargo synthesis procedure suing 75 kD and 300 kD poly-D-lysine backbone materials respectively.

Synthesis of butoxy carbonyl-protected non-covalent fluorophore cargo (BOC-cargo)

4.0 mg HBr salt of D-lysine chain polymer (150 kD) was dissolved in 5 mL methanol followed by adding 100 µL triethyl amine. This mixture was stirred for 30 minutes at 4°C followed by addition of 2.0 mg NHS-fluorescein dye. The resulting reaction mixture was further stirred for 12 h at 4°C. Next, excess BOC-anhydride (5.0 mg) was added to it and the reaction mixture was stirred for another 2 h. The precipitate obtained from this reaction was separated and washed with cold acetonitrile and dried at room temperature under vacuum to get the BOC-protected non-covalent fluorophore cargo.

Synthesis of magnetic bead linked cargo

4.0 mg of each non-covalent and covalent fluorophore cargo was dissolved in 5 mL methanol by adding 100 µL triethyl amine. The reaction mixture was stirred for 30 minutes at 4°C to make free all the amine groups. Next, 1.0 mg of NHS-magnetic beads was added to each of the reaction mixture and stirred for 12 h at 4°C. Un-reacted triethyl amine was neutralized by dropwise addition of ice cold 1 (N) HCl. The brown-yellow precipitate obtained from this reaction was separated and washed with cold acetonitrile and dried at room temperature under vacuum to get the magnetic bead linked respective cargos.

General procedure for cell-surface functionalization and live cell imaging
Jurkat T cells (1×10^5) were plated in a 12 well plate and treated with 100 µg/mL concentration of the cargos in growth media. The cell and cargo mixture were shaken at 120 rpm using an orbital shaker for 30 minutes at room temperature. Next, cells were stained with Hoechst 33342 (for nucleus) in growth media and washed with sterile PBS and transferred in glass bottom dish. Cells were viewed under 60X oil object (optical zoom 3) in confocal laser microscope (Nikon AR1-MP).

Fixed cell surface conjugation and imaging

Jurkat T cells were fixed by 37 ºC pre-warmed 4% paraformaldehyde (15710, Electron Microscopy Sciences) for 15 minutes and immediately transferred in glass bottom dish (MatTek Corporation). Cells were seeded as well as fixed by centrifugation at 1000 rpm at 10ºC for 5 minutes. Next, fixed cells were gently rinsed with PBS to remove any fixation agent and treated with the covalent fluorophore cargo (0.1 mg/mL) for 30 minutes in PBS at RT. Cells were stained with DAPI (4′,6-diamidino-2-phenylindole) and washed with PBS and again centrifuged to make sure their attachment on the glass bottom surface. Finally, confocal images were captured using 60X oil object.

Legends for 3D confocal movie S1 (Cell surface conjugation of Jurkat T cell with 150 kD cargo). Jurkat T cells were treated with the covalent cargo (0.1 mg/mL) for 30 minutes in growth media at room temperature and stained with Hoechst 33342 for nucleus. Cells were then washed with PBS and confocal laser microscopic images were captured in z-stack using 60X oil object. Nucleus is shown in blue and cell surface is shown in green.

Legends for 3D confocal movie S2 (Cell-cell conjugation of Jurkat T cells with 150 kD cargo). Jurkat T cells were treated with the covalent cargo (100 µg/mL) for 30 minutes in growth media at room temperature and stained with Hoechst 33342 for nucleus. Cells were then washed with
PBS and confocal laser microscopic images were captured in z-stack using 60X oil object. Nucleus is shown in blue and cell surface is shown in green.

**Stability of the conjugated cargo molecules on surface modified Jurkat T cells**

Surface conjugated Jurkat T cells (100,000 cells/well) were grown in 12 well culture plate in growth media and images were captured after 1, 3 and 6 days of incubation using confocal laser microscope. Fluorescence intensity was measured using NIS-Elemental software.

**Viability assay of the surface modified Jurkat T cells**

The cell viability experiment was performed using the CellTiter Blue (CTB) reagent. Surface conjugated Jurkat T cells (1×10^5) were seeded in each well of 96-well plates using growth media and incubated in a humidified incubator at 37 °C and 5% CO\textsubscript{2} atmosphere. At the end of the incubation, cell titer blue reagent (10 µl) was added directly to each well and the plates were incubated for additional 3 h at 37 °C to allow cells to convert resazurin to resorufin, and the fluorescent signal was measured at 590 nm after exciting at 560 nm using a multiplate ELISA reader (Bio-Tek Synergy HT plate reader, Bio-Tek, Winooski, VT). The percentage of live cells in a cargo-conjugated sample was calculated by considering the fluorescence intensity of the vehicle treated un-conjugated Jurkat T cell sample as 100 %.

**Cargo displacement reactions with surface modified Jurkat T cells in presence of no fluorophore-tag cargo molecules**

Jurkat T cells were conjugated with the non-covalent and covalent fluorophore cargos for 30 minutes. Next, these surface conjugated Jurkat T cells (100,000 cells/well) were taken in 12 well culture plate and treated with no fluorophore tag non-covalent and the dual-conjugation covalent cargo for another 30 minutes in growth media at 120 rpm shaking at room temperature. Cells were treated with Hoechst 33342 for nuclear stain and washed with PBS. Finally, cells were transferred
in glass bottom wells and confocal images were recorded to monitor the retention of surface conjugation.

Reversibility and reusability of the covalent cargo reagent

Jurkat T cells were taken in 24 well plate and incubated with magnetic bead linked covalent fluorophore cargo reagent for 30 minutes at 120 rpm on an orbital shaker. The excess cargo and surface conjugated cell mixture were centrifuged at 500 rpm for 2 minutes to decant the ‘unconjugated cargo’ from the top of the well. Next, surface conjugated Jurkat T cells were taken in 24 well plate and incubated with PBS of pH 5.5 for 30 minutes at 120 rpm on an orbital shaker. The magnetic bead linked free cargo molecules were isolated by magnetic separation from its T cell mixture. The recovered cargo solution thus obtained was adjusted to pH 7.0 by adding 1 (N) NaOH solution and re-used with combination of unconjugated cargo to incubate live Jurkat T cells for another 30 minutes at 120 rpm. The precipitated Jurkat T cells obtained in each step were stained with Hoechst for nucleus and washed with PBS to remove any trace of cargo reagent. Confocal images were acquired using 60X oil object.

T-cell proliferation (manufacturing): T-cell culture in presence of the cargo reagent

Jurkat T cells \((1 \times 10^5)\) were seeded in 96-well plates in growth media and incubated for 3 days in presence of 10 \(\mu\text{g/mL}\) concentration of these cargos in a humidified incubator at 37°C and 5 % CO\(_2\) atmosphere. At the end of the incubation, cell titer blue reagent was added directly to each well and the plates were incubated for 3 h at 37°C to allow cells to convert resazurin to resorufin, and the fluorescent signal was measured using a multiplate ELISA reader (Bio-Tek Synergy HT
plate reader, Bio-Tek, Winooski, VT). The percentage of live cells in a cargo reagent treated sample was calculated by considering the vehicle treated Jurkat T cell sample as 100%.

**Proliferation and clustering of the Jurkat T cells in presence of the cargo reagent (IncuCyte live-cell analysis).**

(2×10^5) cells/well were taken in 48-well plates in growth media and treated with 10 μg/mL of the cargos and incubated for 6 days at 37 °C inside the IncuCyte incubator. Both the proliferation and clustering of the Jurkat T cells were monitored by real time image and video recorded by IncuCyte S3 live cell analysis system. After 6 days of treatment, the clustered Jurkat T cells in each well were treated with PBS (pH 5.5) for 10 minutes at 120 rpm in an orbital shaker. Next, images of the HCl treated cells were recorded again by the IncuCyte S3 live cell analysis system. Finally, magnetic cell separation technique was employed to isolate cargo free pure Jurkat T cells.

**IncuCyte movie S3:** Jurkat T cell and vehicle treatment

**IncuCyte movie S4:** Jurkat T cell and cargo 2 treatment
Figure S1. **Small molecule treatment with Jurkat T cells.** Small molecule fluorophore Fluorescein and its ADP conjugate internalize inside the Jurkat T cells – indicating the necessity of cargo molecule for cell surface conjugation. Scale bar is 5 μm.
Figure S2. Design strategies and structures of cargo molecules for live cell surface chemical conjugation. (a) Design of the noncovalent and dual-conjugated covalent cargo backbone with both cationic side chain and phosphoric acid functionality. (b) Structures of the non-covalent and covalent cargo molecule, and (c) phospholipid linked non-covalent and covalent cargo.
Figure S3. Synthesis of non-covalent and covalent phospholipid cargos. 3-sn-phosphatidic acid sodium salt (from egg yolk lecithin) was used as the source of phospholipid in these reactions.
Figure S4. Synthesis of covalent cargo for live cell surface conjugation. Step wise synthetic schemes for the phosphate (ADP) linked covalent cargo. First, carboxylic group of iodoacetic acid was activated with benzotriazole and the activated [iodoacetic acid intermediate] was reacted with the cationic side chain polymer (150 kD) to get cargo intermediate I (iodoacetic acid intermediate was used as ¼th equivalent with respect to total amine groups of the polymer chain. Smallest possible unit structure was shown in this step, but multiple amide bond formation might occur in adjacent positions). Cargo intermediate I was further reacted with adenosine di-phosphate (ADP) in presence of base di-methyl amino pyridine (DMAP) to get the covalent cargo (ADP was used as ¼th equivalent with respect to total amine groups of the polymer chain, so that 50 % of the amine groups remain as free in the covalent cargo. As the nitrogen lone pair of the amine group in the adenine is less nucleophilic due to its extended conjugation with the aromatic system, therefore in presence of base, either 3'-OH or 2'-OH of adenine forms bond with the cargo intermediate I. Smallest possible unit structure of the covalent cargo was shown in this step).
Figure S5. Synthesis of non-covalent and covalent fluorophore cargos. The non-covalent cargo polymer (150 kD) was reacted directly with NHS-fluorescein in presence of triethylamine to get the non-covalent florescent cargo. (NHS-fluorescein was used as \(\frac{1}{4}\)th equivalent with respect to total amine groups of the polymer chain in non-covalent cargo, so that 75 % amine groups remain as free. Similarly, NHS-fluorescein was also used as \(\frac{1}{4}\)th equivalent with respect to total amine groups of the polymer chain in covalent cargo, so that 50 % amine groups remain as free. Smallest possible unit structure was shown in each step.)
Non-covalent fluorophore cargo  Covalent fluorophore cargo

Nucleus

Cargo

Merge

5 μm 5 μm
Figure S6. Surface conjugation of Jurkat T cells with 150 kD non-covalent and covalent fluorophore cargos. Cells were treated 0.1 mg/mL concentration of the cargo for 30 minutes and images were captured using 60X object in confocal laser microscope. Scale bar is 5 µm.

Figure S7. Cargo concentration optimization for cell-surface conjugation. Cargo concentration 0.01 mg/mL showed very low yield of surface conjugation while cargo concentration 0.1 mg/mL displayed suitable conjugation as well as less precipitation of excess cargo (for 1.0 mg/mL) during the conjugation and washing procedure. These data revealed cargo
concentration 0.1 mg/mL was suitable for such conjugation reactions. Cells were viewed using 60X objective in Cytation 5 imaging reader. Scale bar is 30 µm.
**Figure S8.** The impact of the structure and molecular weight of cargos on live cell surface functionalization and its cytocompatibility. (a) Structure of the cargo 1 (75 kD), 2 (150 kD) and 3 (300 kD). (b) Confocal laser microscopy images showing Jurkat T cell surface conjugation with these cargos 1-3. Scale bar 5 µm. (c) Cytocompatibility of Jurkat T cells after surface conjugation with the cargos 1-3. Among these molecules, cargo 2 showed better efficiency of surface conjugation as well as cytocompatibility. Results are expressed as mean ± SD. *p < 0.05 stands for a statistically significant difference compared with the no cargo control group and ‘ns’ stands for statistically non-significant.

**Figure S9.** Fixed cell membrane imaging application. Cell-surface imaging of Jurkat T cells using the covalent cargo reagent (0.1 mg/mL, Scale bar is 5 µm).
Figure S10. Confocal laser microscopic images of the surface-modified Jurkat T cells conjugated with both the cargos after 1, 3, and 6 days. Scale bar 5 μm.
Figure S11. Surface conjugation of Jurkat T cells with BOC-protected cargo. Ammonium cationic side chain protected cargo did not show any surface conjugation of Jurkat T cells – indicating the plausible role of this group in surface bond formation. Scale bar is 5 µm.

Figure S12. Surface conjugation of activated human T cells. Live cell surface imaging of IL-2 activated Jurkat T cells. Expected surface conjugation was observed for the both non-covalent and covalent cargo treatments. Scale bar is 5 µm.
Figure S13. Probing additional cell surface bonding interaction between covalent and non-covalent cargos. (a) Surface conjugated Jurkat T cells were treated with non-covalent cargo without fluorophore tag for. (b) Surface conjugated Jurkat T cells were treated with covalent cargo without fluorophore tag. Decreased green fluorescence intensity was observed for the non-covalent cargo conjugated T cells while it was less for the covalent cargo conjugated T cells. Scale bar is 5 µm.
Figure S14. Schematic representation showing synthesis of magnetic bead linked covalent fluorophore cargo and its recovery. (a) Synthetic step for the covalent fluorophore magnetic bead cargo using NHS-magnetic beads. (b) Experimental steps showing separation of unconjugated magnetic cargo for reusable application. (c) Experimental steps showing isolation of the magnetic cargo reagent from the surface conjugated T cells. After disruption of surface conjugation, the free cargo molecules were isolated by magnetic separation and used as reusable cargo for further surface conjugation of live cells.
Figure S15. Reversibility and reusability of the cargo reagent for live cell imaging. (a) Confocal images of surface conjugated Jurkat T cells with covalent cargo reagent. (b) Jurkat T cells after disruption of surface conjugation by pH stimulation. (c) Surface conjugation of Jurkat T cells with the combination of washing and recovered cargo reagent. Scale bar is 5 µm.
Figure S16. **Cell-cell conjugation through cargo linker.** Bright field images of unconjugated and cargo conjugated Jurkat T cells captured in Cytation 5 imaging reader. Scale bar 30 µm.
Figure S17. Bio-manufacturing of therapeutic T cells and their magnetic cell separation. IncuCyte real time images of live Jurkat T cells in presence of the vehicle and cargo 2 after 0- and 6-day treatments. More confluence and clustering of Jurkat T cells (as marked with yellow circle) were observed for the treatment of the cargo 2 as compared to that of the no cargo treated Jurkat T cells. Magnetic cell separation was used to isolate free cells from its cargo mixture.
**Figure S18.** $^1$H-NMR spectrum of the non-covalent cargo in D$_2$O at RT.

**Figure S19.** $^1$H-NMR spectrum of the cargo intermediates I in D$_2$O at RT.
Figure S20. $^1$H-NMR spectrum of the **covalent cargo** in D$_2$O at RT.

Figure S21. $^1$H-NMR spectrum of the **non-covalent phospholipid cargo** in D$_2$O at RT.
Figure S22. $^1$H-NMR spectrum of the covalent phospholipid cargo in D$_2$O at RT.

Figure S23. $^1$H-NMR spectrum of the non-covalent fluorophore cargo in D$_2$O at RT.
Figure S24. $^1$H-NMR spectrum of the covalent fluorophore cargo in D$_2$O at RT.
Figure S25. FT-IR spectra of (a) **phospholipid fatty acid** (ester C=O band at 1741.22 and P=O band at ~1170.61 cm\(^{-1}\)), (b) **non-covalent cargo** (amide C=O band at ~1697.92 cm\(^{-1}\)), and (c) **non-covalent phospholipid cargo** (amide C=O band at 1685.37 and P=O band at ~1156.14 cm\(^{-1}\)).
Figure S26. FT-IR spectra of (a) adenosine di-phosphate (P=O band at ~1103.40 cm\(^{-1}\)), (b) covalent cargo (amide C=O band at ~1692.45 and P=O band at ~1075.56 cm\(^{-1}\)) and (c) covalent phospholipid cargo (amide C=O band at ~1698.73 and P=O band at ~972.86 cm\(^{-1}\)).
References

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