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Lipophilic indocarbocyanine conjugates for efficient incorporation of enzymes, antibodies and small molecules into biological membranes

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Abstract

Decoration of cell membranes with biomolecules, targeting ligands and imaging agents is an emerging strategy to improve functionality of cell-based therapies. Compared to covalent (e.g., click) chemistry or genetic expression on the cell surface, lipid painting (i.e., incorporation of lipid-conjugated molecules into the cell bilayer) is a fast, non-damaging and less expensive approach. Previous studies demonstrated excellent incorporation and retention of distearyl indocarbocyanine dye DiI in membranes of cells *in vitro* and *in vivo*. In order to exploit the membrane stability of DiI, we synthesized an amino-DiI derivative, to which we subsequently conjugated an antibody (cetuximab), an enzyme (superoxide dismutase), and a small molecule (DyLight 800). Red blood cells have long been used as drug delivery vehicles so they were utilized as a model to study the incorporation of DiI conjugates in the plasma membrane. All the DiI constructs demonstrated fast and efficient *ex vivo* incorporation in the membrane of mouse RBCs, resulting in millions of exogenous molecules per RBC. Following an intravenous injection into mice, the molecules were detected on circulating RBCs for several days. DiI anchored molecules showed longer residence time in blood and significantly higher area under the curve (AUC) compared to free non-conjugated molecules. Thus, cetuximab, SOD and DyLight painted on RBC showed 5.5-fold, 6.5-fold and 78-fold increase in the AUC, respectively, compared to the non-modified molecules. Lipophilic indocarbocyanine anchors are a promising technology for

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incorporation of biomolecules and small molecules into biological membranes for *in vivo* applications.

Keywords

DiI; erythrocyte; membrane; painting; SOD; antibody; near infrared fluorescence

INTRODUCTION

Improving the pharmacokinetics (prolonging circulation time and delaying hepatic and renal clearance) of biologics and drugs is of great importance to the field of drug delivery. To meet this end, several strategies have been developed, including nano-formulations [1], PEGylation [2], and fusion with polypeptides [3]. An alternative approach is to piggyback molecules on biological structures that exhibit inherent prolonged circulation, such as albumin and the Fc portion of antibodies [4, 5], and red blood cells [6–9]. Due to their inherent biocompatibility and long-circulation times, RBCs are especially interesting for delivery of genes, chemotherapy, contrast agents and enzymes [10–14]. Several strategies to append molecules to the RBC surface have been tested, including covalent modifications [15], antibodies to integral RBC surface proteins [8, 16], and genetic expression of sortase and subsequent ligation [17].

In many of the above cases, modifications of RBCs are difficult to control and scale up, whereas affinity tags can be competitively displaced *in vivo*. We have previously explored a strategy to “paint” the plasma membrane of RBCs with antibodies for *in vivo* targeting to leukocytes and lymphoma cells [18, 19]. In this approach, antibodies were covalently conjugated to distearoyl phosphatidylethanolamine (DSPE) via a PEG₃₄₀₀ linker and then incubated with RBCs, which resulted in the incorporation into the membrane. However, the incorporation efficiency was relatively low (~16,000 IgG per RBC). Therefore, there is a need in alternative approaches to lipid painting, using anchors that exhibit better affinity for biological membranes. Previously we have found that very efficient membrane painting can be achieved with lipophilic indocarbocyanine dye DiI, which is a distearyl analog of Cy3 fluorescent dye. Painted RBCs showed excellent retention of DiI after incubation in serum *in vitro* and after several days in circulation *in vivo* [18, 19]. Other reports also confirm that lipophilic indocarbocyanine dyes (e.g., DiO, DiI, DiD) have excellent *in vivo* membrane retention [20, 21]. Indeed, these dyes have been historically used for labeling cell membranes and *in vivo* neuronal tracking [22]. Some of the stability in the bilayer could be attributed to a highly lipophilic nature of the dyes and mild cationic charge on the indole ring, allowing them to deeply embed in the negatively charged RBC bilayer [23].

Here, we set out to capitalize on the membrane affinity and stability of DiI and explore its use as an anchor for the incorporation of various molecules into the cell membrane. To this end, we synthesized an amino DiI derivative where an aminomethyl moiety is attached to the 6 position of the indole ring to facilitate covalent attachment of biomolecules and small molecules. Our studies demonstrate efficient *ex vivo* painting and good *in vivo* retention of the molecules in the biological membrane of RBCs. To this end, we demonstrated prolonged

circulation of a variety of cargo molecules, including an antibody, an enzyme and a small molecule. We used mouse RBCs as a model biological membrane, but the utility of the dyes can be extended to other cell types. Lipophilic indocyanine dyes are promising candidates for lipid painting of biological membranes with bioactive molecules.

RESULTS

In order to use DiI as the membrane anchor, we first synthesized a methylamine DiI derivative as described in Methods. This derivative was subsequently reacted with NHS-PEG₃₄₀₀-maleimide to afford a thiol reactive Mal-PEG₃₄₀₀-DiI. To determine the ability of the construct to paint biological membranes, the maleimide group of Mal-PEG₃₄₀₀-DiI was blocked with glutathione (Fig. 1A) to prevent covalent modification of surface proteins, and then incubated with murine erythrocytes for 30 min at 37°C in the presence of a small amount of fetal bovine serum (which we found empirically to improve the painting efficiency). RBCs showed efficient painting with DiI-PEG as verified by fluorescent microscopy (Fig. 1B). The painted RBCs were then injected intravenously into a BALB/c mouse. Flow cytometry analysis of blood samples taken over the course of 7 days showed excellent stability of painted RBCs (Fig. 1C), with over 60% of the injected RBCs still present in the circulation at 7 days (Fig. 1D).

To demonstrate the applicability of DiI as a membrane anchor for antibodies, we labeled mouse IgG with near infrared fluorescent dye IRDye 800 (hereafter IRDye), thiolated with Traut's reagent and coupled it to Mal-PEG₃₄₀₀-DiI (Fig. 2A). The yield of the conjugation was ~60% as verified by gel electrophoresis (Fig. 2B). Painting of RBCs with IRDye-IgG-PEG₃₄₀₀-DiI showed a linear correlation with the concentration in the loading buffer (Fig. 2C), and the loading yield was $16.9 \pm 0.9\%$. This corresponds to 2.2 million IgG molecules per RBC at 5 μ M concentration in the loading buffer. This loading efficiency is much higher than what was previously found for IgG-PEG₃₄₀₀-DSPE (~16,000 IgG/RBC [18]). In order to test functionality of the antibody on the RBC surface, we painted RBCs with anti-mouse Fc-specific IgG-PEG₃₄₀₀-DiI construct and then added IRDye labeled mouse IgG. Anti-Fc RBCs, but not control RBCs, efficiently captured the fluorescent IgG (Fig. 2D). Next, we determined if all IgG is localized on the outer membrane of RBCs. RBCs were painted with IRDye-IgG-PEG₃₄₀₀-DiI, incubated in a protease cocktail (trypsin + proteinase K), washed and analyzed for intact IgG with SDS-PAGE. As shown in Supplemental Fig. 1, fluorescent IgG progressively disappeared from RBCs over time, with no IgG left on RBCs at 24h, suggesting that the entire antibody is exposed to the protease. Lastly, we determined the effect of DiI anchor on circulation of IRDye-modified IgG *in vivo*. Despite the long-circulating properties of engineered antibodies, heavy modification with small molecules and drugs alters their pharmacokinetic properties [24]. Thus, accelerated clearance is the primary factor that constrains the number of drugs that can be conjugated to an IgG molecule, limiting the potency of drug antibody conjugates [24, 25]. We modified anti-EGFR antibody cetuximab with ~9 IRDye molecules/IgG. In this case, IRDye was used as both a drug mimetic and a fluorescent reporter. IRDye-cetuximab-PEG₃₄₀₀-DiI was synthesized as described in Methods, painted onto RBCs and injected into a cohort of 3 BALB/c mice via tail vein. Non-lipidated IRDye-cetuximab was injected into another cohort of 3 mice and blood samples were taken at various time points over the course of two days.

The RBC-tethered IRDye-cetuximab showed two-phase elimination, with over 20% of the initial concentration still in circulation 2 days post-injection, whereas free IRDye-cetuximab was almost completely eliminated after 1 day (Fig. 2E). The RBC-anchored cetuximab showed 5.6-fold higher AUC than the free cetuximab (Table 1).

RBC based delivery of enzymes is a promising approach for acute and chronic disease therapy [12, 13, 16, 26, 27], with one emerging application as a trigger of immune tolerance to adhered cargo [8, 28]. To demonstrate the ability of DiI to serve as a membrane anchor for enzymes, we conjugated the sulfhydryl reactive Mal-PEG₃₄₀₀-DiI to thiolated superoxide dismutase (SOD). SOD is a naturally occurring enzyme in mammalian cells that breaks down highly reactive superoxide radicals that can lead to cellular damage [29]. SOD has been considered for enzyme replacement therapy against oxidative damage caused by radiation therapy and reperfusion injury [30, 31]. Unfortunately, the enzyme has poor retention in the blood stream and is therefore of limited use in the clinic unless it can be tethered to a more stable platform *in vivo* [32]. IRDye was conjugated to the enzyme and used as a reporter to determine SOD concentration in blood. The fluorescent enzyme was thiolated using Traut's reagent and coupled to Mal-PEG₃₄₀₀-DiI forming IRDye-SOD-PEG₃₄₀₀-DiI (Fig. 3A). SDS-PAGE analysis indicated an overall yield over 60% (Fig. 3B). In order to determine the efficiency of RBC loading, various concentrations of IRDye-SOD-PEG₃₄₀₀-DiI were titrated in the loading buffer. Measurement of infrared fluorescence of washed painted RBCs revealed linear dependency on incubation concentration. On average, $9.9 \pm 0.3\%$ of the IRDye-SOD-PEG₃₄₀₀-DiI in the loading buffer became stably bound to erythrocyte membranes (Fig. 3C), and each RBC had ~ 1.3 million SOD molecules at 5 μM IRDye-SOD-PEG₃₄₀₀-DiI loading concentration.

Next, we assessed *in vivo* stability of SOD-painted RBCs. The effect of membrane tethering on the pharmacokinetics of SOD was determined by tail vein injection of IRDye-SOD-PEG₃₄₀₀-DiI painted RBCs into a cohort of 3 mice. Separately, 3 mice were injected with an equal amount of IRDye-SOD. Blood samples were collected at various time points, lysed in SDS buffer and blotted on nitrocellulose for NIR fluorescence analysis. Without the anchor, SOD fluorescence was rapidly cleared from the blood (less than 10% of initial blood fluorescence in 30 min) whereas levels of SOD anchored to RBCs remained above 10% for 4 days (Fig. 3D). SOD anchored to RBCs showed 6.5 fold higher blood AUC than free SOD (Table 1). In order to confirm that the SOD is attached to RBCs after injection, we performed flow cytometry analysis of whole blood after systemic injection. For this purpose, we labeled SOD with Cy3, and prepared Cy3-SOD-PEG₃₄₀₀-DiI construct. We painted mouse RBCs as described above and injected into a BALB/c mouse. As shown in Fig. 3E, the percentage of Cy3-SOD-PEG₃₄₀₀-DiI positive RBCs in the blood stream remained relatively constant for two hours post-injection and decreased by about 30% at 24 h. The SOD fluorescence decreased by 45% during this time period. These experiments suggest that Cy3-SOD-PEG₃₄₀₀-DiI-painted RBCs are stable *in vivo*, but the construct is gradually removed from the RBC surface.

Finally we set out to determine the efficiency of RBC membrane painting with small molecules. To this end we employed DyLight 800 (hereafter DyLight), a hydrophilic NIR dye. The dye was directly conjugated to amino-DiI via an NHS ester (Fig. 4A and Methods).

Similar to the previously described constructs, the loading efficiency of DyLight-DiI showed linear dependency on the concentration in the loading buffer (Fig. 4B) and the yield was 60%, which is surprisingly high considering the expected repulsion between the negatively charged dye and the anionic RBC membrane. Each RBC on average contained 7.8 million molecules of the dye at 5 μ M loading concentration. The painting resulted in RBCs being fluorescent in both Cy3 and DyLight channels (Fig. 4C). We injected mice with either free DyLight, free DyLight-DiI or DyLight-DiI-painted RBCs and measured the circulation *in vivo*. According to Fig. 4D, the fluorescence in blood of mice injected with DyLight was completely gone 1 h post-injection whereas RBC bound DyLight was at nearly 60% of its initial concentration. Four days after injection 25% of the initial DyLight-DiI on RBCs was still in the circulation, whereas there was none of the free dye. Moreover, DyLight-DiI on RBCs showed over 5-fold higher levels than free DyLight-DiI. The RBC-anchored dye showed 77.7-fold higher AUC than free dye (Table 1). At day 4 post-injection, we scanned the organs of the mice for NIR fluorescence using Li-COR Odyssey. DyLight was retained in the tissues of the mice that had been injected with DyLight-DiI painted RBCs and free DyLight-DiI, but was nearly undetectable in the organs of the mice that had been injected with DyLight (Fig. 4E). Interestingly, the distribution of fluorescence in organs was different for free DyLight-DiI and DyLight-DiI painted on RBCs. For the painted RBCs, the main accumulation organs were spleen, liver and femur, whereas for DyLight-DiI, the main accumulation organs were lung, kidney, femur and liver, whereas spleen showed much lower deposition (Fig. 4F).

To test if painting of RBCs with the DiI constructs triggers hemolysis and complement activation, we used SOD-PEG₃₄₀₀-DiI or cetuximab-PEG₃₄₀₀-DiI (5 μ M in RBC loading buffer). After washing three times to remove unbound constructs, we incubated RBCs with mouse serum for 1.5 h at 37°C (non-painted RBCs were used as control) and measured the absorbance of the supernatant at 550 nm. Painting RBCs with SOD or cetuximab resulted in a minimal lysis compared to control RBCs (Supplemental Fig. 2). Complement C3 opsonization assay showed 6% increase for SOD-painted RBCs and 51% increase for cetuximab-painted RBCs compared to plain RBCs. It appears that this increase in C3 opsonization did not cause excessive hemolysis, but potentially could lead to the accelerated clearance of cetuximab-painted RBCs.

Finally, we investigated the self-assembling properties of DiI derivatives. For this purpose, we measured fluorescence intensity of DiI, DiI-amine and DiI-PEG as a function of increasing concentrations. Since fluorescent dyes are known to undergo self-quenching, the DiI fluorescence is expected to decrease when critical aggregation concentration (CAC) is reached. The constructs showed a very different aggregation behavior. Thus, DiI fluorescence showed linear increase in the tested range of concentration (10 nM to 10 μ M), suggesting that its CAC is likely below 10 nM. On the other hand, DiI-amine and DiI-PEG showed CAC at $\sim$ 0.87 μ M and $\sim$ 0.88 μ M, respectively (Fig. 5A). In the presence of 1% fetal bovine serum, there was a tendency for decreased aggregation, judging by the increase in fluorescence for DiI amine and DiI-PEG (Fig. 5A). To confirm the effect of FBS on aggregation properties of DiI-PEG, we measured sizes of fluorescent aggregates using nanoparticle tracking analysis (Nanosight). According to Fig. 5B, the size of DiI-PEG aggregates was very heterogeneous, with mean diameter of 449 nm. After addition of FBS,

the mean diameter decreased to 397nm. Since the RBC loading concentrations are on the order of 5–10 μ M, the DiI constructs should be in the aggregated form, with or without FBS, but FBS could induce less aggregated species that have a better chance of incorporation into the RBC membrane. Since most of the constructs are on the outer RBC membrane, It is unlikely that whole aggregates fuse with the membrane. We hypothesize that the painting process involves partial dissociation of the aggregates in the presence of FBS and incorporation of individual constructs in the RBC membrane (Fig. 5C).

DISCUSSION

Using the novel DiI lipid anchor, we demonstrated a highly efficient method of painting biological membranes with biologics and small molecules. We demonstrated that each RBC could be loaded with over 2 million antibodies, 1 million enzymes and 7 million small molecules. We have found that using DiI as a membrane anchor is a major improvement over phospholipid constructs, which yields only a few thousand ligands adhered to each RBC [18, 19]. RBCs are an attractive experimental platform to test attachment of exogenous molecules due to the ongoing effort to convert RBCs into drug carriers and immunomodulators [33]. Several strategies have been described to attach targeting ligands and enzymes to RBCs, including genetic expression, covalent attachment, affinity ligands and lipid painting [8, 12, 13, 15, 34]. All these methods have their advantages and disadvantages, among the critical factors being the extent of RBC damage, ease of modification, and retention on RBC membrane. In our opinion, lipid painting has the advantage of stable incorporation in the bilayer without the need for covalent modification, but with the potential tradeoff of ex vivo modification and processing of blood. Since there are examples of successful ex vivo modification of donor RBCs for use in patients in the clinic [35–37], we envision that the painting step can be scaled up for potential clinical use.

RBCs painted with DiI anchors did not show hemolysis and complement activation in vitro. Some echinocytosis (formation of small spikes on RBCs) was observed (Fig. 1), but this was not a consistent observation. Echinocytosis can be caused by stress conditions, changes in pH, Ca²⁺, membrane composition [38–40], and is usually reversible, for example after addition of serum [40]. PEG-DiI-painted RBCs showed long circulation times in vivo (Fig. 1), suggesting that the anchor insertion causes minimal damage to the cells. DiI anchor significantly prolonged the tested payloads' circulation in blood, which was one of the goals of piggybacking molecules on the RBC surface. Molecules that were otherwise rapidly cleared were retained in circulation for days. At the same time, all the constructs tested showed a biphasic elimination profile. We previously demonstrated that the initial elimination of phospholipid-tagged IgG and fluorescein was mostly due to the loss from the RBC surface [19]. Several lines of evidence suggest that this is also the case with the DiI constructs. First, the initial loss of the SOD from circulation was much faster than the drop in levels the painted RBCs (Fig. 3). This suggests that the initial elimination phase is due to the transfer of the lipidated constructs to other compartments. Second, PEG-DiI showed much slower loss than SOD-PEG-DiI, suggesting that the molecule attached to the DiI anchor dictates the transfer. Bulky IgG and SOD constructs showed much faster elimination than the small molecule DyLight. One possibility is that RBC-appended molecules transfer to endothelial and sinusoidal membranes, which was shown previously for GPI anchored

proteins [41] and phospholipid-anchored antibodies [19]. In addition, molecules like IgG can be captured via immunoglobulin receptors on liver and spleen macrophages [42]. Since our data show that DiI constructs interact with serum proteins (Fig. 5A), it is possible that some proteins, including albumin, trigger removal of the constructs in circulation. We did not investigate the histological localization of the payload in the body. Interestingly, DyLight-DiI showed differences in the distribution of organ fluorescence between free construct and RBC-anchored construct. In particular, the lung signal was much lower in case of RBC-anchored than free construct, suggesting less efficient transfer.

Several strategies can be envisioned to further improve functionality of the DiI constructs. The ideal constructs should exhibit fast membrane association kinetics and reduced self-aggregating properties. The stability in the membrane can be improved by using longer chain lipids instead of stearyl C18. At the same, increasing hydrophobicity may decrease the critical micelle concentration, leading to diminished incorporation efficiency. Our ongoing efforts are focused on understanding the balance between the construct structure and incorporation and retention efficiency. An interesting venue of exploration would be to conjugate molecules to lipids via cleavable linkers, for example protease sensitive linkers that are cleaved when exposed to the microenvironment associated with disease [43].

Finally, we used mouse RBCs for their apparent utility in drug delivery. Since RBCs can be considered a model biological membrane, we envision that the utility of the DiI anchors can be extended to other cell types. For instance, cell-derived particles and stem cells show unusually high affinity for the diseased tissues [44–46]. Modification of these particle and cells with targeting ligands and other molecules can enhance their functionality and open new venues for research. Another direction could be a fast and economical modification of immune cells with targeting ligands and drugs for immunotherapy applications [47].

METHODS

Synthesis of Aminomethyl-DiI (scheme 1):

Step 1: 2 mL of 2,3,3-Trimethylindolenine **1** (1.984 g, 0.013 mol, 1 eq.) and 8.19 g of 1-Iodooctadecane (0.022 mol, 1.75 eq.) were dissolved in 4.2 mL of chloroform and heated at reflux for 96 hours. The solvent was then removed under reduced pressure and the resulting dark red residue was washed multiple times with hexanes and ether to give 5.0712 g of indolium **2** as a dusty reddish brown solid.

Step 2: 3 mL of 2,3,3-Trimethylindolenine **1** (2.98 g, 18.7 mmol, 1.1eq.) and 3.01 g of N-(Hydroxymethyl)phthalimide **3** (16.93 mmol, 1 eq.) were added to 14.7 mL of concentrated sulfuric acid and stirred at room temperature for 6 days. The reaction was then poured over ice and made basic with concentrated aqueous ammonium hydroxide. After crystallizing at –8°C overnight, the resulting precipitate was filtered, rinsed with ice cold water, and dried under vacuum to give indolenine **4** as a light yellow shiny crystalline solid.

Step 3: 5.5 g of indolenine **4** (17.3 mmol, 1 eq.) and 11.5 g of 1-Iodooctadecane (30.2 mmol, 1.75 eq.) were dissolved in 5.5 mL of chloroform and stirred at reflux for 5 days. The solvent was then removed under reduced pressure and the residue was washed, filtered, and

rinsed with hexanes and diethyl ether to give **5** as a dark red solid. The crude product was used in the next step without further purification.

Step 4: 0.98 g of **5** (1.71 mmol, 1 eq.), 0.70 g of N,N'-Diphenylformamidine **6** (3.42 mmol, 2 eq.), and 0.4 mL of triethyl orthoformate (0.356 g, 2.14 mmol, 1.2 eq.) were dissolved in 5.1 mL absolute ethanol and stirred at reflux. The reaction was monitored by TLC using 10% methanol in DCM as solvent, and was complete after 22 hours. The solvent was then removed under reduced pressure and the resulting red residue was purified by preparatory HPLC using a gradient of 65–100% acetonitrile in water (+ 0.1% TFA) with detector absorbance set to 411 nm. Hemicyanine **7** was isolated as 565 mg of a bright yellow solid (0.84 mmol, 49% yield).

Step 5: 1.13 g of hemicyanine **7** (1.68 mmol, 1 eq.), 1.39 g of indolium 2 (3.36 mmol, 2 eq.), and 1.6 mL acetic anhydride (1.73 g, 16.8 mmol, 10 eq.) were dissolved in 8.4 mL of pyridine and heated at 60°C. The reaction was monitored by TLC (10% methanol in DCM) and was complete after 2 days. The solvent was azeotroped with toluene and removed under reduced pressure to give cyanine **8** as a dark purple residue. For characterization, a small amount of this material was purified by semi-preparatory HPLC using a gradient of 65–100% acetonitrile in water (+0.1% TFA). The rest of the material was used crude in the next step.

Step 6.—500 mg of cyanine **8** (0.503 mmol, 1 eq.) was dissolved in 2 mL of methanol, and then 1.5 mL of anhydrous hydrazine (1.53 g, 47.8 mmol, 95 eq.) was added slowly. The reaction was stirred at room temperature for 2 days and monitored by TLC (10% methanol in DCM). The solvent was then evaporated under reduced pressure and the resulting dark pink residue was purified by prep HPLC using a gradient of 85–100% acetonitrile in water (+0.1% TFA) to give 289 mg of pure cyanine **9** as a bright pink solid (0.33 mmol, 67% yield).

¹H NMR (400 MHz): δ = 8.42–8.49 (t, J=16 Hz, 1 H), 7.71 (s, 1 H), 7.53–7.55 (d, J=8 Hz, 1 H), 7.42–7.46 (t, J=8 Hz, 2 H), 7.30–7.34 (t, J=8 Hz, 1 H), 7.14–7.17 (q, J=4 Hz, 2 H), 6.08–6.12 (dd, J=16 Hz, 2 H), 4.19 (s, 2 H), 3.99 (m, 4 H), 1.82 (m, 4 H), 1.74 (s, 12 H), 1.45–1.39 (d, J=24 Hz, 8 H), 1.25 (s, 55 H), 0.85–0.89 (t, J=8 Hz, 6 H).

C₆₀H₁₀₀N₃ (M⁺): m/z = 862.792, found 862.796.

Mal-PEG3400-Dil synthesis (Scheme 2):

15 μmol (10 nm) of aminomethyl-DiI **9** was combined with 37.5 μmol (135 mg, 2.5 eq) NHS-PEG3400-Maleimide in DMSO with 5 eq (with respect to amino-DiI, 10 μL) TEA and allowed to react for 1.5 h at room temperature. The product **10** was purified on an Isolera purification system (Biotage, LLC, Charlotte, NC, USA) using a Biotage SNAP Ultra C18 30 g column with a 30–95% methanol-water gradient (0.1% TFA). The product was dried under vacuum with an overall yield of 10%. The product was verified by mass spectrometry.

Superoxide Dismutase (SOD)-PEG₃₄₀₀-DiI Synthesis: SOD (58 μ M, 50 μ L) in PBS (pH 7.4) was reacted with 25 μ g either IR Dye 800 CW NHS or sulfo-cy3 NHS. After 2 h incubation at room temperature, excess dye was removed by size exclusion chromatography (7 kD cutoff Thermo Scientific Zebra column). Thiolation was achieved with a 30-fold excess of Traut's reagent along with 2.5 mM EDTA in PBS. Excess Traut's reagent was removed by size exclusion chromatography. A 2.5 fold excess of mal-PEG₃₄₀₀-DiI in DMSO (final concentration less than 5%) was added and the mixture was allowed to react for 48 h at 4°C. The synthesis was confirmed by SDS PAGE analysis on a Bio-Rad Mini-PROTEAN TGX Stain-Free 4–20% acrylamide gel. Densitometry revealed an overall yield of 60%.

IgG-PEG₃₄₀₀-DiI Synthesis: Mouse IgG (10 mg/mL, 50 μ L) in PBS (pH 7.4) was reacted with 25 μ g IRDye800CW NHS. After 2 h incubation at room temperature, excess dye was removed by Zebra column). Thiolation was achieved with a 30-fold excess of Traut's reagent along with 2.5 mM EDTA in PBS. Excess Traut's reagent was removed by size exclusion chromatography. A 2.5 fold excess of mal-PEG₃₄₀₀-DiI (DMSO) was added and the mixture was allowed to react for 48 h at 4°C. The synthesis was confirmed by SDS PAGE analysis on a Bio-Rad Mini-PROTEAN TGX Stain-Free 4–20% acrylamide gel. Densitometry revealed an overall reaction yield of 60%.

For cetuximab-PEG₃₄₀₀-DiI synthesis, ERBITUX (2 mg/mL, 100 μ L) was solvent exchanged into PBS (pH 7.4) using a Zebra spin column and reacted with 75 μ g IRDye800CW NHS. After 2 h incubation at room temperature, excess dye was removed by size exclusion chromatography. UV-Vis spectral analysis revealed an average of 9 dyes conjugated per antibody. Thiolation was achieved with a 30-fold excess of Traut's reagent along with 2.5 mM EDTA in PBS. Excess Traut's reagent was removed by size exclusion chromatography. A 2.5 fold excess of mal-PEG₃₄₀₀-DiI (DMSO) was added and the mixture was allowed to react for 48 h at 4°C. The synthesis was confirmed by SDS PAGE analysis on a 4–20% acrylamide gel. Densitometry revealed an overall reaction yield of 17%. Lower lipidation was the result of high dye conjugation rate.

DyLight 800-DiI synthesis (Scheme 3):

952 nmol (1 mg) of DyLight 800 NHS ester was combined with 750 nmol aminomethyl-DiI **9** in 100 μ L dry DMSO with a 5 fold excess of triethylamine. The solution was maintained at room temperature in the dark for 5 h. Excess DyLight 800 was removed by 5x

dichloromethane: PBS extraction (1:10) keeping the organic layer. The structure **11** was confirmed by an Applied Biosystems 4800 Plus MALDI-TOF/TOF Analyzer mass spectrometer. $C_{105}H_{148}N_3 + 3H$ (M^+): $m/z = 1754.04$, found 1754.04.

Erythrocyte Loading: Whole blood was obtained from adult BALB/c mice. The blood was washed three times in PBS by centrifugation at 1500 g being careful to remove the buffy coat. The erythrocyte pellet was combined with various concentrations of DiI constructs and 2% FBS in PBS at a volume ratio of 2:1:1. The maximum concentration of the constructs was determined experimentally as the one that does not induce RBC hemolysis. The mixture was incubated at 37°C for 30 min, and RBCs were washed 3 times in PBS. To determine loading efficiency, RBCs were bath sonicated and loaded in 1 μ L triplicates on a 0.22 μ m nitrocellulose membrane (Bio-Rad). The membrane was scanned at 800 nm using Li-COR Odyssey (for near infrared labeled constructs) or at 565nm using Bio-Rad gel camera (for DiI). The spot integrated density of a 16-bit TIFF image was measured with ImageJ and plotted as a function of time with Prism (GraphPad, San Diego CA). Concentrations were calculated using a standard curve for the respective constructs. Labeled RBCs were imaged with Zeiss Axio Observer 5 epifluorescent microscope equipped with X-Cite 200DC light source and AxioCam 506 monochromatic camera. Near infrared labeled RBCs were imaged with Cy7 filter set, catalog number 49007, Chroma Corporation (McHenry, IL, USA).

Monitoring of RBCs in Circulation: To monitor levels of painted RBCs at different times post-injection, flow cytometry was used as described before [18]. PEG₃₄₀₀-DiI (200 μ M, 100 μ L in PBS) was treated with 1 mM glutathione for 1 h. After this time a shift of around 300 Daltons was observed in Gaussian distribution of the mass spectrum of PEG₃₄₀₀-DiI by MALDI, confirming the maleimide had been inactivated. Fresh washed RBCs (40 μ L) were painted as previously described using 50 μ M thiolated PEG₃₄₀₀-DiI in the loading buffer. The pellet was washed and then injected into a BALB/c mouse, which was bled at 30 min, 1 d, 2 d, and 7 d post injection. Blood samples were diluted to less than 3% hematocrit

in PBS and analyzed on a flow cytometer (FACScan, BD) in FL-2 channel. The percentage of DiI-positive RBCs was determined. The same protocol was used for monitoring of SOD painted RBCs.

For monitoring the circulation of NIR-labeled molecules, RBCs were painted as previously described. RBCs or controls (NIR-labeled molecules without DiI anchor) were injected into the tail vein of BALB/c mice and blood was collected at various time points via retro orbital bleeding. After blood was diluted two-fold with lysis buffer (0.1% SDS in H₂O), samples were dotted in triplicate on a nitrocellulose membrane (2 μ L) and the fluorescence intensity was measured with a LiCOR infrared scanner at 800nm wavelength.

Compliment Hemolysis Assay: Blood was withdrawn from a BALB/c mouse and washed three times to obtain a RBC pellet. 10 μ L of pellet was incubated with either with 5 μ M IRDye800-SOD-PEG₃₄₀₀-DiI or IRDye800-Cituximab-PEG₃₄₀₀-DiI as described above. After painting, 10 μ L RBCs were mixed with 30 μ L mouse serum and incubated at 37°C for 1.5 h without shaking. Then, the RBCs were briefly pelleted, and the absorbance in the supernatant was measured by UV-Vis spectrometry at 550 nm. The absorbance of hemoglobin in control supernatant (non-loaded RBCs incubated in the same conditions) was considered 100%, and the hemolysis of painted RBCs was expressed as percentage of control.

Complement C3 Opsonization: Blood was withdrawn from a BALB/c mouse and washed three times to obtain a RBC pellet. 5 μ L of pellet was incubated with either with 5 μ M IRDye800-SOD-PEG₃₄₀₀-DiI or IRDye800-Cituximab-PEG₃₄₀₀-DiI as described above. After painting, 5 μ L RBCs were mixed with 15 μ L mouse serum and incubated at 37°C for 1.5 h without shaking. Then, the RBCs were washed 3x and resuspended at 30% hematocrit and lysed by sonication. The lysates were blotted on a nitrocellulose membrane and probed with goat anti-mouse C3 antibody (Quidel Inc, San Diego CA) and an anti-goat secondary antibody labeled with IRDye 800 (Li-COR Biosciences, Lincoln, NE). The blot was analyzed on a Li-COR Infrared Scanner Odyssey.

Critical Aggregation Concentration Determination: The aggregation was determined by monitoring the fluorescence of the lipophilic anchor at different concentrations in the presence or absence of FBS. Titrations (10 μ M to 0.0025 μ M) of PEG-DiI, amino-DiI, and DiI were dissolved in either PBS or PBS with 1% FBS (to mimic loading buffer conditions). These solutions were transferred to a 96 well plate (200 μ L). DiI fluorescence was read on a SpectraMax M5 (Ex: 520 nm, Em: 575 nm, cutoff: 550 nm) at maximum sensitivity. The critical micelle concentration was determined from the negative peak value of the first derivative of the resulting fluorescent curve (Prism 6.0 software).

Size Measurements: Lipophilic aggregates were analyzed on a NanoSight NS300 (Malvern Instruments, Malvern, UK) using fluorescent imaging mode with 488 nm laser. The fluorescence wavelength, albeit not optimal for DiI, was still able to detect the fluorescent aggregates. PEG-DiI (10 μ M) was dissolved in either PBS or PBS with 1% FBS. Data represent 5 separate measurements.

Surface Digestion: To demonstrate that cargo is loaded exclusively onto the cell exterior. IR-IgG-PEG₃₄₀₀-DiI was loaded onto a red blood cell pellet (80 μ L) as previously described. An aliquot of labeled RBCs was saved to determine the initial fluorescent intensity of the pellet. Corning 0.25% Trypsin, 2.21 mM EDTA (160 μ L, in cell culture medium) was added to the loaded pellets, along with 200 μ g/mL proteinase K (Fluka). RBCs were incubated at 37°C and agitated at 800 rpm on an eppendorf thermomixer R. The solution was gently vortexed and 15 μ L aliquots were removed at different time points up to 90 min. After this, the reaction was placed at 4°C and allowed to continue for 24 h, when the final aliquot was taken. Aliquots were washed 2x in PBS, monitoring for RBC lysis to ensure proteases had no access to RBC interior. Pellets were lysed (2 μ L) in 0.1% SDS (18 μ L) and blotted on a nitrocellulose membrane and analyzed by LiCOR or by SDS-PAGE for the disappearance of IgG over time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Stability of PEG-DiI painted RBCs *in vivo*.

A) Structure of the GST-PEG₃₄₀₀-DiI construct. B) Erythrocytes were painted with GST-PEG₃₄₀₀-DiI and detected with fluorescence microscopy in the FL-2 channel; C) Painted RBCs were injected into a BALB/c mouse. Flow cytometry analysis at different time points shows that painted RBCs are stable in peripheral blood. The graph shows the percentage of painted RBCs cells in the peripheral blood and also the mean fluorescence of painted RBCs.

Figure 2. IgG-PEG-DiI painted RBCs:

A) IgG (cetuximab) was labeled with IRDye 800 and thiolated Traut's reagent. The modified antibody was conjugated to Mal-PEG₃₄₀₀-DiI as described in Methods; **B)** Reducing SDS PAGE was used to characterize the antibody constructs. Heavy and light chains of cetuximab are shown with black arrows. Antibody-DiI constructs were compared to native antibody. The additional mass of the PEG₃₄₀₀-DiI adducts resulted in shifted bands (blue arrows). The heavy chain appears to be far more reactive than the light chain. **C)** RBCs were painted with fluorescent cetuximab and the loading efficiency of IgG was determined by measuring the antibody recovered from washed red blood cells after the painting process. The loading efficiency was similar for all concentrations tested (~17%). Inset shows microscopic image of IgG-PEG-DiI painted RBCs; **D)** To demonstrate that IgG painted on RBC surface remains functional, RBCs were painted with anti-mouse Fc-specific IgG-PEG-DiI as described above and then mixed with IRDye800 labeled mouse IgG. RBCs were washed, lysed and the fluorescence was measured with near infrared scanner Odyssey. Anti-Fc antibody efficiently captured the labeled antibody whereas control RBCs did not retain

any fluorescence; E) IgG (cetuximab)-painted RBCs were injected intravenously. Mice were bled at different time points and NIR fluorescence in blood spots was determined by dot blot analysis as described in Methods. The negatively charged NIR molecules on IgG (9 IRDye/Ab) leads to poor circulation of the free antibody which was cleared within 1 day, whereas the labeled IgG painted on RBCs shows prolonged circulation (20% of the initial concentration at 48 hours). P-value of 2-sided t-test, N=3.

Figure 3. Stability of Superoxide Dismutase SOD-PEG₃₄₀₀-DiI painted RBCs *in vivo*:
A) SOD was thiolated with Traut's reagent and conjugated to Mal-PEG₃₄₀₀-DiI; **B)** Reduced SDS PAGE was used to characterize the constructs. The different bands (blue arrows) correspond to the DiI linker modified SOD; **C)** RBCs were painted with IRDye-SOD-PEG-DiI and the loading efficiency of the enzyme was determined as described in Methods. The painting efficiency was similar for all concentrations (~10%); **D)** RBCs were painted with IRDye-SOD-PEG₃₄₀₀-DiI and injected intravenously. Control mice were injected with IRDye-SOD. Mice were bled at different time points and NIR fluorescence in blood spots was determined by dot blot analysis as described in Methods. Painting SOD on RBCs resulted in higher SOD levels in blood and much higher AUC (Table 1) compared to free SOD. Thus, the majority (93%) of non-anchored enzyme had been cleared by 30 min, whereas 23% of the initial concentration of the anchored enzyme remained in circulation after four days. The increase in the level of fluorescence toward the end of the study could

be due to degradation of the fluorescent enzyme and release of fluorescence in the circulation. P-value of t-test, $N=3$.; E) Painted RBCs were injected into a BALB/c mouse. Flow cytometry analysis at different time points shows that the painted RBCs are still in circulation after 24 h post-injection. The graph shows the percentage of painted RBCs cells in the peripheral blood and also the mean fluorescence of painted RBCs.

Figure 4. Stability of DyLight-DiI painted RBCs *in vivo*:

A) DyLight-DiI construct was synthesized as described in Methods; **B)** RBCs were painted with DyLight-DiI and the loading efficiency of the dye was determined as described in Methods. Loading efficiency was over 60% for all concentrations tested; **C)** microscopic image of double-labeled RBCs; **D)** RBCs were painted with DyLight-DiI and injected intravenously. Control mice were injected with free DyLight. Free dye was cleared within 1 hour whereas the dye still circulated bound to RBCs (P-value of 2-sided t-test, N=3). AUC of painted dye was much higher than free dye (Table 1); **E)** Following injection, mice were sacrificed and the organs were scanned in a 12 well plate with Li-COR at 800 nm. Fluorescent images of organs of mice sacrificed 96 h post-injection show significant organ deposition of the dye in painted RBC group. Free dye did not show any deposition probably due to high clearance rate. Quantification of mean fluorescence intensity shows significant differences in spleen, kidney, lung and liver accumulation between DyLight-DiI and DyLight-DiI painted RBCs (P-values of 2-sided t-test, N=3).

Figure 5. Mechanism of lipid painting of cell membrane with DiI constructs.

A) Different concentrations of DiI constructs were prepared microplate wells in PBS or in 1% FBS/PBS. For DiI, fluorescence intensity was independent of concentration and FBS. DiI amine and DiI-PEG showed critical aggregation concentration of $\sim 0.83 \mu\text{M}$ (found with first derivative, Prism software). FBS caused dequenching of the fluorescence suggesting partial disassembly; B) Nanosight measurements of DiI-PEG show heterogeneous sizes of aggregates, and some decrease in size after addition of FBS; C) at the painting concentrations (above critical aggregation concentration), the constructs form large assemblies (e.g., sheets, discs, liposomes). These assemblies are partially disintegrated in the presence of FBS, and the constructs are incorporated into the outer layer of the RBC membrane.

Table 1.
Area under the curve (AUC) of different cargoes (in arbitrary units)

The values were calculated using corresponding blood elimination profiles (Fig. 3,4,5) using Prism software.

Cargo	RBC-anchored	Free
IRDye-cetuximab (at 24h)	960	173
IRDye-SOD (at 96h)	1714	263
DyLight (at 72h)	2308	29.7