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Precision Labeling of Native Antibodies with Lock Coupling

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Abstract

The formation of stable protein complexes enables much of biotechnology, but even high-affinity complexes can dissociate, limiting potential applications in biomaterials development, bioimaging, nanomedicine, and other protein-based technologies. Here, we describe Lock coupling, a simple and selective one-step reaction between interfacial lysine and glutamate or aspartate sidechains to form stable isopeptide bonds and be used for the precise labeling of native antibodies. We identify conditions in which short-lived activated esters formed by the aqueous carbodiimide EDC promote isopeptide bond formation specifically at pre-associated amine-acid pairs. Indiscriminate crosslinking is minimized by formation of protein complexes before addition of catalyst, use of acidic pH to suppress exposed Lys reactivity, and limiting the aqueous stability of activated esters. For native antibody (Ab) labeling, we show that the small IgG-binding protein GB1 can be covalently attached to the Ab Fc domain, and introduction of Cys into GB1 loops allows for facile conjugation of fluorophores, micelles, or inorganic nanocrystals for imaging in live cells and animals. By varying Cys substituents and protein stoichiometry, a defined number of probes can be uniformly attached without the need for extensive purification. In live-cell confocal microscopy, labeled GB1 serves as a stable replacement for secondary Ab, enabling simple multicolor immunostaining and imaging. Lock coupling requires just a single reagent in aqueous buffer and leverages both the innate ability of proteins to form high-affinity complexes and the widespread presence of Lys-Glu/Asp pairs at their interfaces, with potential for precision synthesis of protein-based probes for imaging, biomaterials, biophysics, and medicine.

Keywords

bioconjugation, proteins, antibody, click, diimide, imaging, fluorescence

Precision Labeling of Native Antibodies with Lock Coupling

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Abstract

The formation of stable protein complexes enables much of biotechnology, but even high-affinity complexes can dissociate, limiting potential applications in biomaterials development, bioimaging, nanomedicine, and other protein-based technologies. Here, we describe Lock coupling, a simple and selective one-step reaction between interfacial lysine and glutamate or aspartate sidechains to form stable isopeptide bonds and be used for the precise labeling of native antibodies. We identify conditions in which short-lived activated esters formed by the aqueous carbodiimide EDC promote isopeptide bond formation specifically at pre-associated amine-acid pairs. Indiscriminate crosslinking is minimized by formation of protein complexes before addition of catalyst, use of acidic pH to suppress exposed Lys reactivity, and limiting the aqueous stability of activated esters. For native antibody (Ab) labeling, we show that the small IgG-binding protein GB1 can be covalently attached to the Ab Fc domain, and introduction of Cys into GB1 loops allows for facile conjugation of fluorophores, micelles, or inorganic nanocrystals for imaging in live cells and animals. By varying Cys substituents and protein stoichiometry, a defined number of probes can be uniformly attached without the need for extensive purification. In live-cell confocal microscopy, labeled GB1 serves as a stable replacement for secondary Ab, enabling simple multicolor immunostaining and imaging. Lock coupling requires just a single reagent in aqueous buffer and leverages both the innate ability of proteins to form high-affinity complexes and the widespread presence of Lys-Glu/Asp pairs at their interfaces, with potential for precision synthesis of protein-based probes for imaging, biomaterials, biophysics, and medicine.

Introduction

Antibody (Ab) conjugates are the foundation of much of biotechnology and modern medicine, and methods for the synthesis of bioactive immunoconjugates have driven advances in imaging, immunotherapies, cell sorting, and cell-specific targeting of drugs and radionuclides. For immunoconjugate synthesis, the simple amide-forming reaction of IgG Lys sidechains with activated esters is widely used for covalent attachment of fluorophores and other biophysical probes, but because each IgG has >80 primary amines, this labeling reaction is wildly heterogeneous, both in the number and the positions of probes.^{1,2} Alternatively, partial IgG disulfide reduction affords free Cys that may be modified, although this also can lead to heterogeneous labeling as well as changes to IgG stability. Lack of synthetic control may present problems in preparation of precisely-defined probes or therapeutics, in weakening Ab affinity for its intended target, or in promoting aggregation and instability.

These limitations have led to the search for simple and precise Ab labeling strategies that are generally applicable for chemically diverse probes. Site-specific Ab-labeling methods have been well developed, particularly for Ab-drug conjugates,^{3–5} and include “hot cysteine” engineering,⁶ expressed aldehyde tags,⁷ and enzymatic modification of peptidyl inserts.⁸ For native Ab, without mutagenesis or other synthetic components, options are much more limited,⁹ and include some types of glycan modification,¹⁰ UV crosslinking,¹¹ and proximity labeling¹² or enzymatic modification¹³ of specific positions.

Among the many naturally occurring proteins that bind to Ab, the B1 domain of streptococcal protein G (GB1) is a small, thermostable protein that binds all 4 classes of human IgG and has found widespread use in a range of Ab technologies.¹⁴ The microbial role of GB1 is apparently to misorient Ab so that they cannot bind to Fc receptors: it binds to each of 2 identical Fc sites at the C_{H2}/C_{H3} interface with nanomolar affinity (K_d values variously reported between 10 and 250 nM),^{15,16} and to Fab domains about an order of magnitude more weakly.¹⁷ The size and stability of GB1 have also made it attractive for studies of protein folding,^{18,19} design,^{15,20} and biophysics.^{21,22}

Salt bridges between amines and acids are common at protein-protein interfaces, with over 70% of interfacial Lys, Glu, and Asp sidechains forming Coulombic or H-bonds between proteins,²³ and serving as keystone interactions or conformational constraints.²⁴ In rare instances, these amine-acid pairs may spontaneously condense into isopeptide bonds,^{25,26} either within or between proteins. Recent functional proteomics studies have suggested isopeptides are more common than their frequency in structural databases,²⁷ as even high-resolution structures may have difficulty distinguishing amine-acid pairs from amide bonds.²⁸ Isopeptide-containing proteins have been reengineered into split protein systems such as SpyCatcher,²⁹ which has been exploited in a variety of labeling^{30–32} and engineering^{33,34} applications.

Inspired by these natural and engineered isopeptide-based conjugation systems, and by pioneering chemical biology of Koshland and others,^{35–40} we describe Lock coupling, a selective one-step reaction

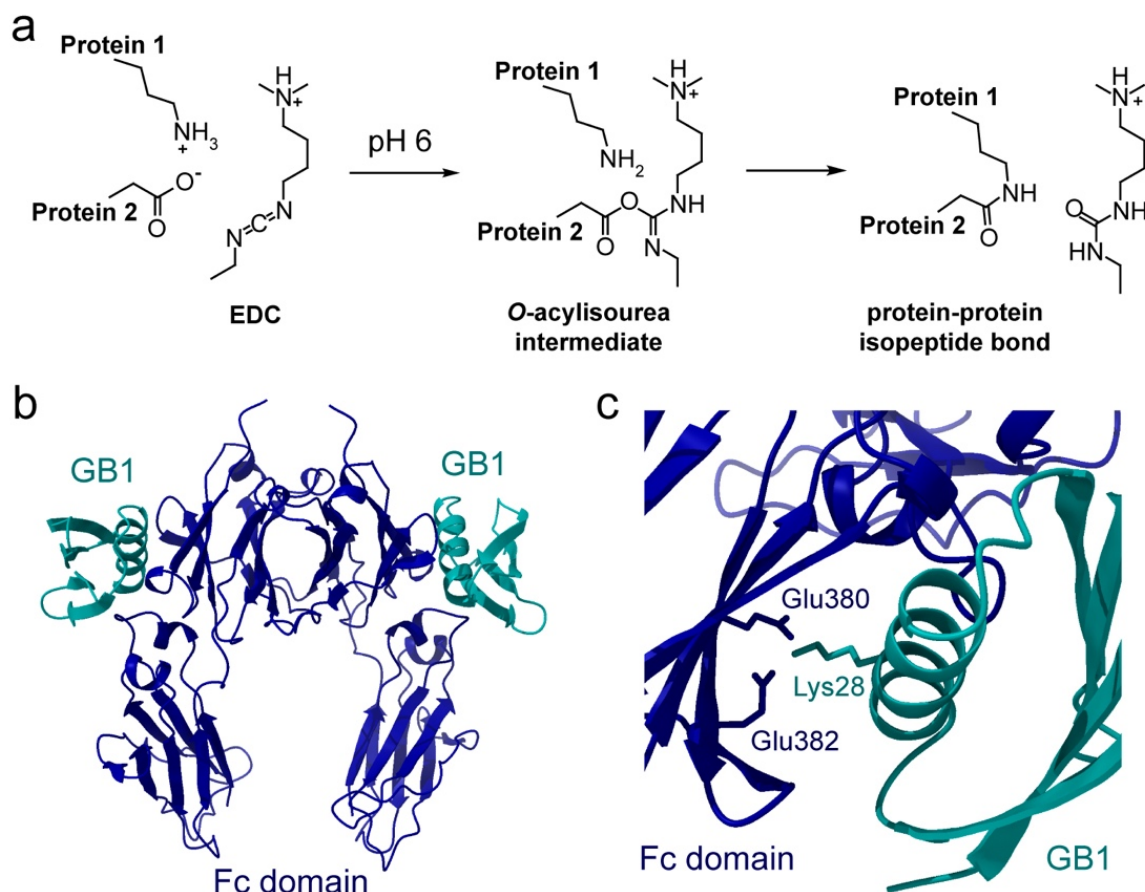


Figure 1. Lock coupling to form interprotein isopeptides from native salt bridges. (a) EDC activation of sidechain carboxylate, followed by nucleophilic attack by neighboring Lys ϵ -NH₂ to form interprotein isopeptide bond. Use of pH 6 in Lock coupling suppresses reactivity of other Lys. (b) Structural model of GB1s bound to human IgG1 at C_{H2}/C_{H3} interfaces.⁴¹ (c) Detail of GB1 Lys28 interactions with conserved IgG1 Glu380 and Glu382.

between native, interacting Lys/Asp or Lys/Glu sidechain pairs, which can form isopeptide bonds between proteins and be used for the precise labeling of IgG Ab. We show that short-lived activated esters formed by the common aqueous cross-linking reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, **Figure 1**) can promote isopeptide bond formation specifically at pre-bound protein-protein interfaces. Indiscriminate crosslinking is minimized by formation of protein complexes before addition of carbodiimide catalyst to create high effective concentrations, use of moderately acidic pH to suppress exposed Lys reactivity, and limiting the aqueous stability of activated esters so that only pre-associated amines can react. For Ab labeling, we show that GB1 can be covalently bound to the IgG Fc domain, and introduction of cysteines into GB1 allows for conjugation of fluorophores, micelles, or alloyed upconverting nanoparticles (aUCNPs). This eliminates the innate heterogeneity of most IgG labeling methods, avoids disruption of Fab binding sites, and enables precise multivalent labeling with a large number of probes, including non-reactive lipophilic fluorophores. Lock chemistry requires just a single reagent in aqueous buffer and leverages both the innate ability of proteins to form high-affinity complexes and the widespread presence of Lys-Glu/Asp pairs at those interfaces.

Results and Discussion

Development of a protein coupling reaction between native sidechains. To find reaction conditions for controlled isopeptide bond formation, we explored inefficient diimide coupling conditions to only allow pre-associated acid-amine pairs to react. Diimides such as EDC activate carboxylic acids to *O*-acylisourea intermediates (Figure 1a), which promote reactions with nucleophiles, most often primary amines. Because *O*-acylisoureas are quickly hydrolyzed in the mildly basic conditions needed to deprotonate ammonium groups,⁴² low-pKa alcohols such as *N*-hydroxysuccinimide have been typically used to extend the aqueous half-lives of activated ester intermediates in the pH range needed for amine reactivity, improving yields and limiting side reactions.⁴³ We therefore explored the effect of pH on EDC coupling of IgG1 Ab with the small Fc-binding protein GB1 (Figure 1b). AlphaFold 3

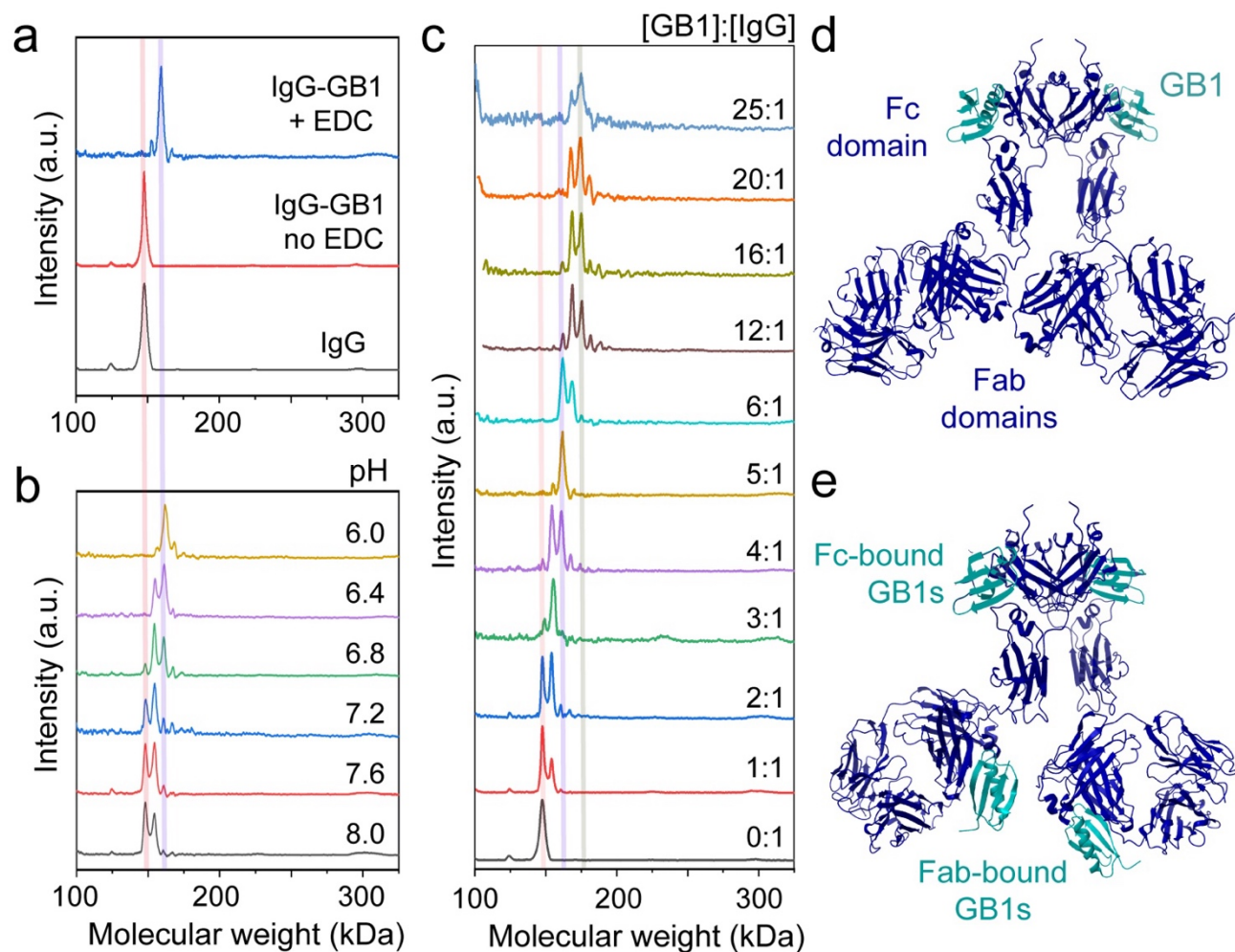


Figure 2. Analysis of IgG-GB1 Lock coupling by mass spectrometry. (a) MALDI of crude reaction mixtures of trastuzumab mixed with a 5-fold molar excess of GB1, with and without EDC. (b) pH dependence of IgG-GB1 EDC coupling, using a 5:1 GB1:IgG molar ratio. (c) Control of stoichiometry by varying GB1:IgG molar ratios, run at pH 6.0. List of mass peaks is in **Table S1**. Vertical lines at 148, 160, and 173 kDa correspond to 0, 2, and 4 GB1s per IgG. (d) Structure of IgG1-(GB1)₂ complexes, with GB1s bound to the Fc domain.^{45,46} (e) Structure of IgG1-(GB1)₄ complexes, with GB1 bound to both Fc and Fab domains.¹⁷

modeling is consistent with X-ray structures of the complex, showing Lys28 of GB1 making close contact with IgG Glu380 and Glu382 in the Fc constant regions (Figure 1c) in each of the 2 identical GB1 binding sites. These Glu are conserved in all human and most mammalian IgG subclasses, as well as all human IgG1 allotypes.⁴⁷

We used matrix-assisted laser desorption/ionization (MALDI) mass spectrometry to screen for reactivity and specificity of EDC-mediated conjugation of GB1 to the α -HER2 IgG1 trastuzumab. Trastuzumab has been reported to show complex glycosylation patterns,⁴⁴ and MALDI spectra show broadened peaks around 148 kDa (Figure 2a). While Lock coupling could be observed by SDS-PAGE (Figure S1), we adopted MALDI for improved quantitation and resolution, and to analyze a mass range able to detect IgG-IgG adducts. Addition of GB1, with or without EDC, shows that only with EDC are there masses larger than IgG alone, corresponding to the 6.4 kDa mass of GB1. Minor, broad

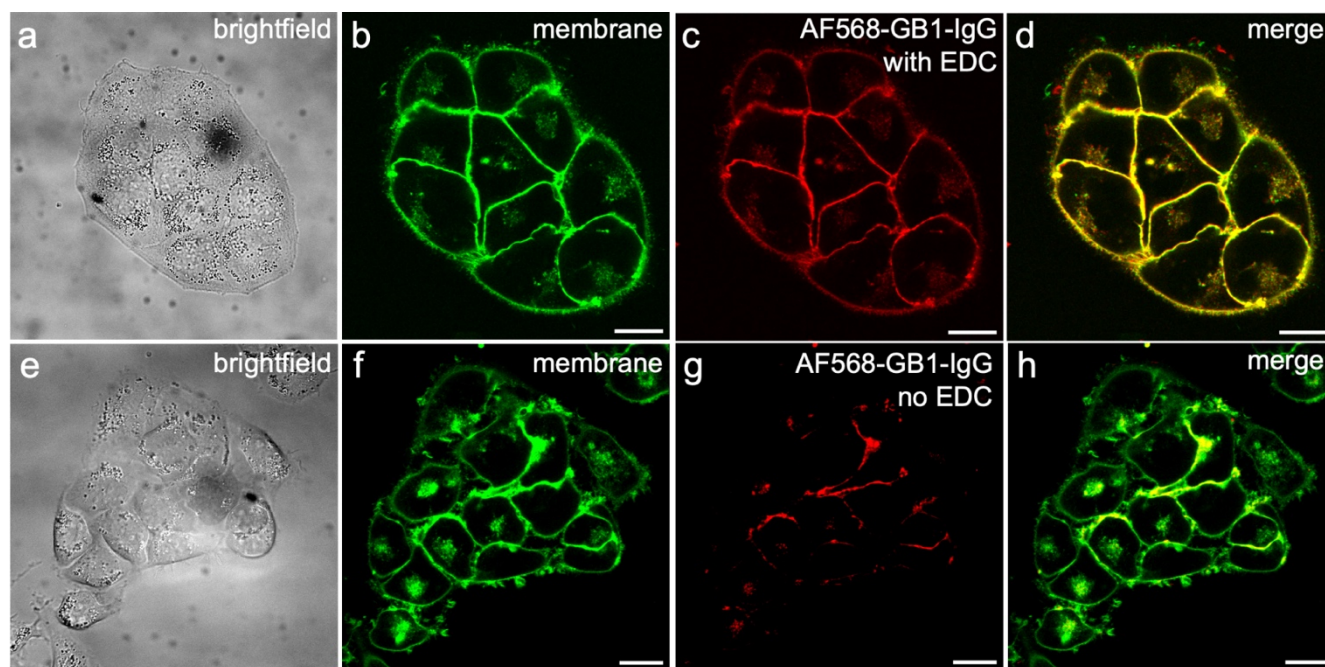


Figure 3. Live-cell imaging with fluorescently labeled GB1-locked IgGs. (a) Brightfield images of a live HCC-1569 cell cluster. (b) Confocal images of cells incubated with (b) Cellmask Green membrane stain, and (c) α -uPAR IgG-(AlexaFluor568)₄ conjugates. (d) Merged fluorescent images. (e-h) as (a-d) above, except IgG complexes with GB1-(AlexaFluor568)₂ are unconjugated by EDC. Scale bars are 20 μ m.

peaks corresponding to IgG-IgG byproduct at ~296 kDa are apparent at high pH but diminish to background at pH < 6.5 (Table S2). Examination of pH within standard physiological range shows the highest efficiency at pH 6.0, with complete conversion of IgG to the bis-GB1 adduct (Figure 2b, Table S1). Reaction of exposed Lys with activated esters is typically run at pH ~ 8; at pH 6, exposed Lys amines are protonated and have no nucleophilic reactivity. This improved efficiency at mildly acidic pH suggests the pK_a of the attacking Lys ϵ -NH₃⁺ group is lowered substantially by the proximity of the EDC tertiary ammonium group, and the *O*-acylisourea group is prone to hydrolysis under neutral conditions. For Lock coupling, it is the hydrolytic susceptibility of the *O*-isoacylurea intermediate that limits reactivity to neutral amines already in its immediate vicinity. Lower pH buffers may also be efficient for EDC coupling, although these may start to destabilize protein-protein interactions, including the GB1-Fc interface.⁴⁸ Conjugation of 1, 2, or 4 GB1s, at both pairs of Fc and Fab binding sites (Figure 2e), is

possible by varying GB1:IgG stoichiometry, although at ratios above 6:1 a loss of specificity becomes apparent, likely because of low-affinity interactions at high GB1 concentrations. While these spectra were taken on crude reactions, additional purification steps will be useful for some applications, and in the experiments below spin dialysis proved sufficient to remove unreacted GB1 and EDC byproduct.

To tune the stoichiometry of this conjugation, we varied the GB1:IgG molar ratio to determine conditions for coupling of either 1 or 2 GB1s per Fc (Figure 2c, Table S1). At higher GB1 concentration,

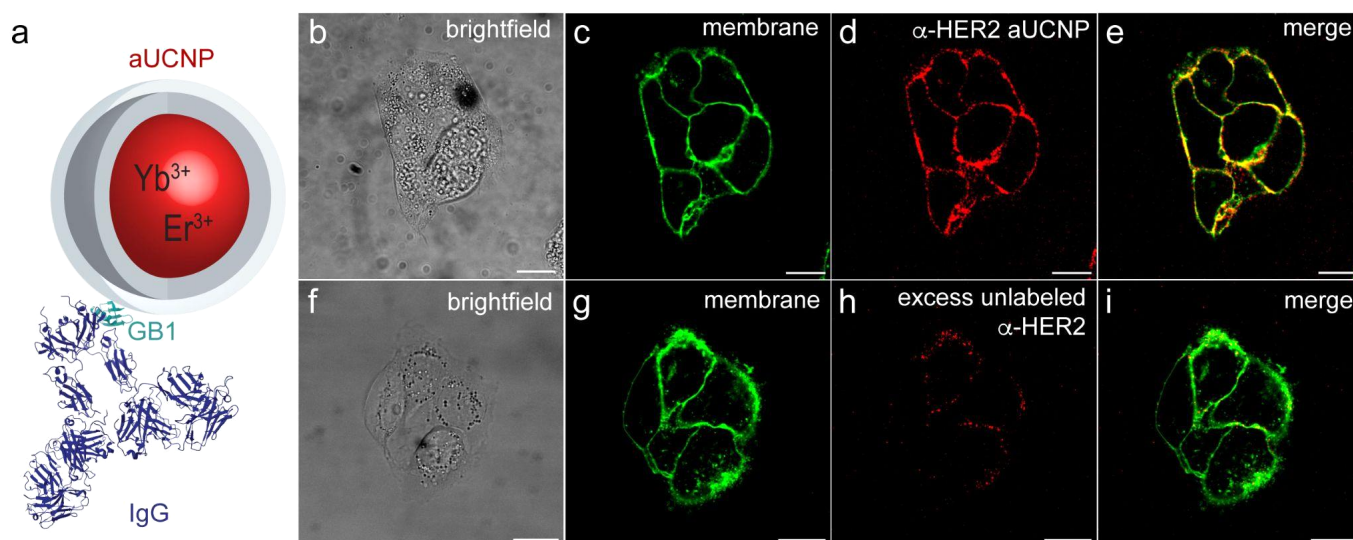


Figure 4. Specificity of EDC-locked immunoconjugates in aUCNP imaging. (a) Structure of IgG-GB1-aUCNP. A 10-nm Yb/Er aUCNP (red) with inert 5-nm NaYF₄ shell (dark gray) and PMAO polymer⁴⁹ (light gray) is disulfide linked to A48C GB1 (teal), then EDC-locked to IgG. Elements depicted to scale. (b) Brightfield and (c-e) confocal images of live HCC-1569 cells incubated with (c) Cellmask orange membrane stain, (d) α-HER2-GB1-aUCNP conjugates, and (e) merged image. (f - i) HCC1569 imaging as in (b - e), except (h) contains a 4-fold molar excess of unlabeled α-HER2 IgG during staining. aUCNP exc: 980 nm; em: 380 - 750 nm. Cellmask Orange exc: 561 nm; em: 575 - 650 nm. Scale bars are 20 μm.

secondary addition at lower-affinity Fab heavy chains is also apparent. At pH 6.0, we find that 3- or 5-fold molar excess of GB1 yields, unpurified, selective formation of either GB1-IgG or (GB1)₂-IgG lock conjugates. Other protein-protein lock conjugates will require similar empirical optimization of stoichiometry or purification schemes.

Live-cell imaging of fluorescent locked immunoconjugates. To test whether Ab conjugated with GB1 retain their activity and specificity, we imaged live breast cancer cells with fluorescently-labeled IgGs specific to the breast and ovarian cancer marker uPAR.⁵⁰ Two Cys point mutations were introduced into GB1 loops facing away from the Fc-GB1 interface and labeled with AlexaFluor568 maleimides (**Figure S2**). To determine if Lock coupling improves receptor staining compared to non-covalent GB1-IgG complexes, we imaged non-EDC-conjugated AF568-GB1-IgG complexes under identical conditions (Figure 3e-h). Without Lock coupling, most of the GB1-IgG complex dissociates during purification (Figure S3), resulting in confocal images with 85% lower integrated intensity (compare Figures 3c and 3g). For a live-cell time-lapse, images were taken after a 20 min incubation at 37 °C, showing membrane staining as well as cytosolic puncta, consistent with prior reports of uPAR internalization to the endolysosomal pathway upon α-uPAR IgG binding.^{30,51} A 2-h time-lapse of a 20-cell cluster (**Supplemental Movie 1**) initially shows well-delineated cell-cell junctions, which become diffuse as

signal is internalized and cytosolic puncta increase and move away from plasma membranes. At ~90 min, trafficking reverses as fluorescence begins to redistribute back toward the membrane, consistent with uPAR recycling mechanisms.⁵¹ These images show that Lock coupling of fluorescent GB1 preserves IgG activity and is necessary for the strong signal needed to image receptor trafficking.

Specific immunotargeting of upconverting nanoparticles. To explore the variety of structures that can be conjugated to IgGs without disrupting their activity, we synthesized immunoconjugates of inorganic nanocrystals, aUCNPs,^{52,53} for upconverted confocal imaging of HCC-1569 cells (**Figure 4**). Hydrophobic aUCNPs were grown as 20 nm NaEr_{0.1}Yb_{0.9}F₄ core/shell heterostructures (**Figures 4a and S4**),⁵⁴ transferred to water by encapsulation within amphiphilic PMAO polymers,⁴⁹ and linked to A48C GB1 *via* an activated disulfide linked to the PMAO.³⁰ EDC crosslinking of a 1:1 ratio of aUCNP to α -HER2 IgG showed growth in diameter of the GB1-aUCNP from 27 to 37 nm by DLS (**Figure S5**) and an average of 1 IgG per aUCNP determined by Trp fluorescence. When applied to live HER2(+) cells, the aUCNP immunoconjugates showed strong membrane localization (**Figure 4d**), co-localizing with a non-specific lipophilic stain and with little apparent internalization (**Figures 4c, 4e**). To test the specificity and affinity of α -HER2 IgGs conjugated to aUCNPs, we performed competition experiments by adding excess unlabeled α -HER2 IgG during staining (**Figures 4, S6, and Table S4**). Loss of integrated

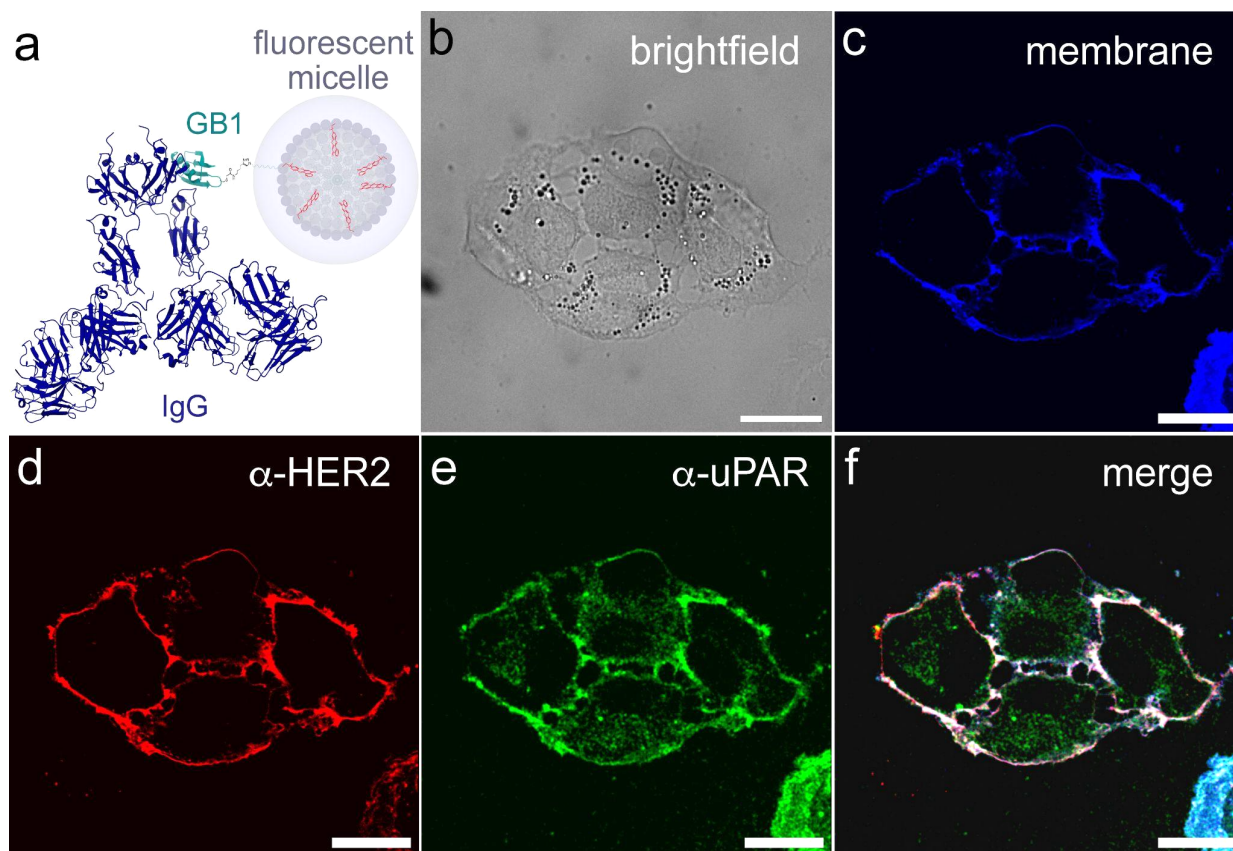


Figure 5. Live-cell confocal microscopy with fluorescent micelle-GB1-IgG conjugates. (a) Structure of EDC-locked IgG-(A48C GB1), conjugated to micelle hosting hydrophobic fluorophores Nile Red or DiD. Click-Lock crosslinking is shown in **Figure S2**. Elements are depicted to scale. (b) Brightfield image, (c) membrane stain Cellmask Green (exc: 488 nm; em: 495 - 550 nm), (d) α -HER2 trastuzumab with Nile Red-loaded micelles (exc: 561 nm; em: 575 - 700 nm), and. (e) α -uPAR IgG with DiD-loaded micelles (exc: 633 nm; em: 640 - 750 nm) of a live HCC-1569 cell cluster. (f) Overlay of fluorescent images. Scale bars are 20 μ m.

emission intensity is linear with increased fraction of unlabeled IgG (**Figure S7**), reflecting no significant change in α -HER2 IgG affinity for HER2 following Lock conjugation to 20-nm nanoparticles.

Click-and-Lock strategy for multi-receptor immunostaining. To demonstrate the specificity and utility of EDC-locked IgGs, we performed 3-color live-cell confocal microscopy on HCC1569 cells with IgGs specific for breast cancer markers HER2 and uPAR (**Figure 5**). A GB1 mutant with an A48C mutation, in a loop on the opposing face of the Fc binding site, was expressed and modified with an alkynyl iodoacetamide for Cu-catalyzed Click conjugation to azide-bearing lipid micelles (**Scheme S1**). GB1-micelles were conjugated using EDC Lock chemistry to either α -HER2 trastuzumab or the α -uPAR IgG 11857.⁵⁰ IgG-GB1-micelles were characterized by dynamic light scattering (DLS), showing average diameters of 28 nm, an increase of 14 nm from GB1-micelles (**Figure S8**), and MALDI showing complete loss of the A48C GB1 peak (**Figure S2**). Micelles were loaded with either Nile Red or DiD, lipophilic fluorophores without conjugation handles (**Figure S9**), by simple addition of fluorophores in DMSO to micelles in buffer, yielding ~6 fluorophores per micelle (**Figure S10**).

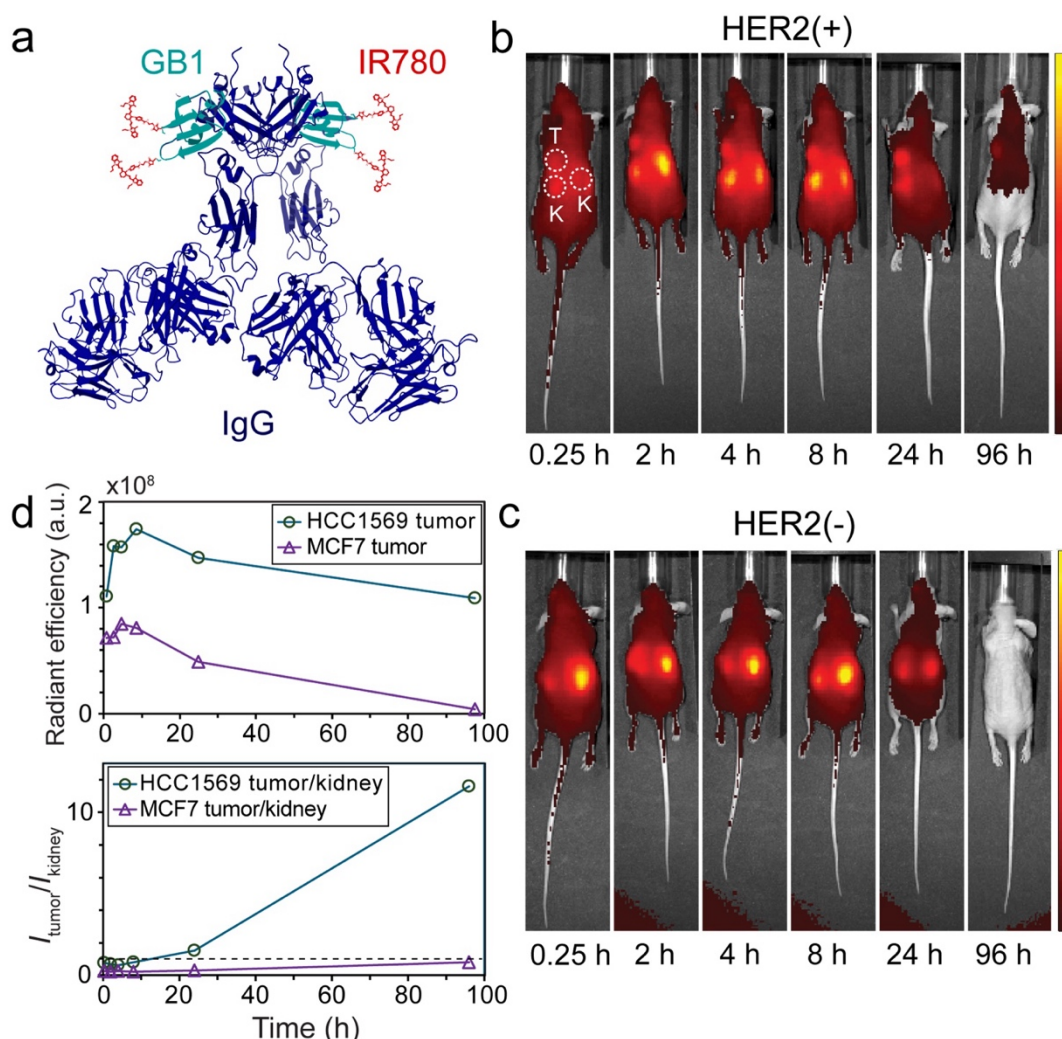


Figure 6. Targeting and imaging of IR780-labeled trastuzumab in live mouse xenografts. (a) Structure of α -HER2 trastuzumab IgG labeled with 4 IR780-HBM fluorophores. (b) Imaging time course of mouse with a HER2(+) HCC-1569 xenograft tumor, injected with NIR780-labeled trastuzumab. T, tumor; K, kidney. (c) Time course of mouse with a HER2(-) MCF-7 xenograft tumor, imaged as in (b). (d) Tumor emission intensity (*top*) and tumor-to-background ratio (*bottom*) of images in (b) and (c).

Addition of both Nile Red-labeled α -HER2 and DiD-labeled α -uPAR IgGs, along with the lipophilic membrane stain CellMask Green, to live HCC1569 cells enables simultaneous imaging of HER2 and uPAR distribution and trafficking patterns (**Figure 5**). After 30 min at 37 °C, HER2 staining tracks closely with non-specific membrane staining and is largely confined to the plasma membrane, while uPAR staining shows the cytosolic puncta of uPAR internalization.^{30,51} The distinct fluorescent patterns of Nile Red and DiD are maintained over 4 h of continuous live-cell imaging, with uPAR internalized in puncta and HER2 remaining largely at the plasma membrane (**Supplemental Movie 2**). These trafficking patterns show that IgG specificity is retained and immunoconjugates show no functional instability under these physiological conditions. Use of lipophilic fluorophores within micelles enables fluorescence amplification without the need for the successive blocking and washing steps needed for secondary Ab, which typically make live-cell multi-color immunostaining difficult.

Live-animal tumor imaging with NIR fluorophore-labeled antibodies. To examine specificity and biocompatibility in live animals, we synthesized trastuzumab labeled with the NIR fluorophore IR780 for imaging in mice with mammary xenograft tumors (**Figure 6**). IR780 was functionalized with a maleimide linker to form Cys-reactive IR780-HBM (**Scheme S2**), which was conjugated to either single- or double-Cys GB1s (**Figure S2**), which were then Locked to trastuzumab, yielding IgGs with 2 or 4 fluorophores. Mice were prepared with xenograft tumors of either HER2(+) HCC-1569 or HER2(-) MCF-7 cells. Emission from injected (IR780)₄-trastuzumab initially appears in dorsal organs of both mice, with signal moving to the HER2(+) tumor over 96 h, with tumor-to-apparent kidney signal reaching 12 (**Figure 6b, 6d**). In the HER2(-) xenograft, little signal appears in the tumor and overall emission is negligible at 96 h (**Figure 6c**). Excised tissue shows similar results, with strong signal in HER2(+) tumors and little to no signal in either HER2(-) tumors or in muscle tissue taken as controls, at both 24 and 96 h (**Figure S11**). This confirms that (IR780)₄-labeled trastuzumab selectively targets HER2(+) tumors and clears from non-target tissues.

Conclusions

We have developed Lock coupling, a 1-step method for selectively coupling native, interacting acid-amine pairs commonly found at protein-protein interfaces. Selective crosslinking at pre-associated Lys/Glu-Asp pairs is achieved by formation of protein complexes before addition of catalyst, use of mildly acidic pH to suppress exposed Lys reactivity, and limiting the aqueous stability of activated esters to prevent diffusive reactivity. These conditions ensure that only carboxylates with nearby amines poised to react can form amides before activated esters are hydrolyzed. Applying Lock chemistry to Ab, we show precise labeling of fully native IgGs with fluorophores, micelles, or inorganic nanocrystals at 1, 2, or 4 positions, with no negative effects on IgG activity, without any mutagenesis or introduction of synthetic components to the Ab. The most likely side reactions are with Lys/Asp and Lys/Glu pairs in loops and turns that have similar proximities as those at protein-protein interfaces. Possible effects of this intra-protein Lock coupling could be detrimental in, for example, altering key allosteric changes, or beneficial, for example by making therapeutic proteins more protease resistant. Other potential applications of Lock coupling, not explored here, could be: new approaches to protein-protein interactomics and *de novo* protein engineering, facilitating preparation of protein complexes for structural studies, and for immunotherapies and analytics with human-derived Ab.

Supporting Information

Supporting Information includes experimental protocols for protein expression and conjugation, fluorophore and nanoparticle synthesis, spectroscopy, modeling, cell culture, microscopy, and analysis; and characterization, imaging, reaction schemes, and tables of mass spectral data; and time-lapse microscopy of fluorescently stained cells (mp4).

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Author Contributions

The manuscript was written through contributions of all authors.

Notes

B.E.C. is an inventor on a patent application covering structures described in this manuscript.

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