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We report synthetic routes for modifying cypate, an indocyanine green fluorophore, with custom thiol-terminated peptide and poly(ethylene glycol) (PEG) substrates. The peptide amino acid sequence serves as a recognition element for the enzyme urokinase plasminogen activator. In collaboration with Dr. Kang (partnering award W81XWH-08-1-0460), peptide, PEG and related spacer conjugates of cypate were attached to nanogold particles for evaluation of their fluorescence properties. We also describe the synthesis of a cypate bis(aldehyde) analog. We developed a new synthetic route to aminooxy- and thiol-terminated hydrophilic-hydrophobic diblock spacers for tethering the cypate bis(aldehyde) to nanogold particles via oximation couplings. In conjunction with a novel aminooxy blocking group (used to modulate cypate polarity), the oximation methodology enabled evaluation of diblock spacer length on cypate fluorescence emission. Finally, we report a synthesis of a tamoxifen-aldehyde for convenient attachment to aminooxy spacers as a means for targeting the nanogold particles.
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**Introduction**

This two-year collaborative project between our and Dr. Kyung Kang’s laboratories (Dept. of Chemical Engineering, Univ. of Louisville) aimed to prepare a gold-based fluorescing nano-entity that could be used for cancer detection and diagnosis. Our goal was to functionalize nanogold particles by linking the fluorescent probe cypate to gold nanoparticles via a cancer enzyme recognition element. Attachment of the probe onto nanogold particles would be engineered such that the fluorescence emission of cypate would be quenched until the nano-entity encountered a cancer-specific enzyme. Encounter with the enzyme, selected as uPA for this study, would result in a selective peptide bond cleavage (linker scission) to release constrained cypate so that its fluorescence emission could occur and would serve as a signal.

To accomplish this goal, we proposed to attach cypate to a nanogold particle using two spacers, a short peptide-based spacer containing a uPA recognition element and a longer, enzyme-resistant poly(ethylene glycol) (PEG) based spacer. As long as cypate is tethered to the nanogold particle surface via the short spacer, its fluorescence emission will be quenched due to the influence of the surface plasmon polarion field of the nanometal. However, after the short spacer is selectively cleaved by uPA, cypate is free to migrate to the length of the long spacer. Migration of the cypate away from the surface of the nanometal not only results in fluorescence emission dequenching but also may result in enhanced fluorescence if the distance of the long spacer is appropriately controlled. Chemistry’s research (our component of the collaboration) thus sought to secure synthetic routes to peptide- and PEG-conjugates of cypate, and to develop efficient methods for their attachment to nanogold particles as well as to optimize the fluorescence properties of the nano-entity; focusing on Task 2 of the Statement of Work, summarized here:

**Task 2.** Construct and test a NGP-cypate conjugate containing a uPA-cleavable site in the short spacer
a. construct the short spacer; attach the spacer to cypate and NGPs, and test the uPA-mediated cleavage;
b. construct the long spacer;
c. conjugate the cypate with short and long spacers onto NGPs and test the performance;
d. conjugate tamoxifen onto the NGPs.

This report summarizes the synthetic progress achieved and observations noted during preparation of the proposed short and long spacers and their respective cypate conjugates. **Data on the optical properties and performance (e.g., response to enzyme exposure) of materials evaluated by Dr. Kang’s group is not included in this report to avoid duplication — see partnering award W81XWH-08-1-0460 for those details.**
A. Synthesis of ‘short spacer’ peptide-cypate conjugate

General considerations. As indicated in our Statement of Work and the Introduction above, we aimed to prepare a peptide-cypate conjugate that (a) can be attached to the surface of a nanogold particle (NGP), (b) is of an appropriate length so that cypate fluorescence emission is quenched by the resident gold surface plasmon field, and (c) can be cleaved by the enzyme urokinase plasminogen activator (uPA), a serine protease synthesized within cancer cells. With these goals in mind, we selected as our target conjugate the thiol-functionalized, peptide-cypate conjugate 1.3 (Scheme 1). The specific amino acid sequence in this prototype follows from the literature that the Gly-Gly-Arg motif is selectively cleaved by uPA (Zimmerman 1978, Chung, 2006) and the length of the peptide chain (6 amino acids plus cysteamine) was chosen based on initial observations by Kang et al. (Kang and Hong, 2006) and subsequent observations by our collaborator Achilefu et al. (Achilefu, 2008), who showed that cypate fluorescence emission was quenched when cypate was attached to a NGP via an 8 amino acid spacer.

Synthesis (Work developed in Yr01). Methoxy-activated, cysteamine-loaded polystyrene resin (Novabiochem) was reacted with the Fmoc-protected amino acids depicted in Scheme 1 following the standard protocol of DIC-mediated coupling and piperidine-mediated deprotection to obtain the resin-bound Boc-protected peptide 1.2. The peptide chain in 1.2 was cleaved from the resin (TFA) and analyzed by HPLC to confirm homogeneity. Several coupling procedures were examined to optimize attachment of cypate to the N-terminus of the peptide chain of 1.2 to minimize the number of cypate equivalents consumed. While polymer site isolation effectively prevented reaction at both carboxylic acid groups of cypate, an excess of cypate (ca. 4 equiv.) was required to achieve modest coupling yields. This result is in agreement with previous reports on synthesis of peptide-cypate conjugates (Fan, 2007; Ye, 2006). We determined that the cypate coupling using DEPBT (Ye, 2005) was most effective (A. Massey, unpublished). Cleavage of the conjugate from the resin was accomplished using minimal TFA in the presence of triethylsilane. Standard cleavage conditions using 50% TFA resulted in cypate decomposition. In general, based on the theoretical concentration of resin reactive sites, conjugate 1.3 was obtained in 8-12% yield (based on total cypate consumed; cleavage yields 60-62%). HPLC purification of 1.3 also exacted a toll on material in that the conjugate appears sensitive to TFA present in the eluent.

Modifications (Yr02). While the approach in Scheme A1 delivered sufficient material (40 mg of peptide-cypate conjugate 1.3 delivered to collaborators in Yr02) to test the validity of cypate quenching (i.e., test short spacer length as well as to probe whether uPA-mediated peptide cleavage can occur in the proximity of the NGP surface — Statement of Work, Task 2a), we believed the design of a route to circumvent hydrophilic peptide (or PEG) conjugate synthesis and purification would constitute a much needed improvement in the experimental design. Along these lines, we developed an oximation strategy for the Yr02 cypate-related couplings (see also sub-sections C & D below).

The reaction between aminooxy (RONH₂) and aldehyde groups (a type of oximation) is a facile, chemoselective coupling that does not require acid- or base-mediated assistance. This reaction proceeds in protic solvent and delivers a robust oxime ether conjugate (RON=C< as the product. We devoted considerable effort toward preparing an aminooxy-terminated analog of the short spacer peptide, aminooxy analogs of PEG-based long spacers, and a bis-aldehyde analog of cypate to actuate the concept of replacing all amide couplings for oximation couplings. These modifications are described below and in the related subsections below.
To underscore the need for development of neutral cypate couplings to short or long spacer molecules, consider a key observation made during the course of these studies: cypate is predisposed to undergo base-mediated (possibly acid-mediated) cleavage reactions (Figure 1). The chromophore of cypate represents a good leaving group; consequently, a $\beta$-elimination of the cypate core from amide analogs (or the acrylic acid sidechain) can occur on treatment with base, such as is present in amide-coupling reactions (e.g. DIPEA). This process may well be facilitated by exposure to silica gel, as normal phase SiO$_2$ chromatography often has led to compound degradation being prevalent. We have also observed mass spectrometry-induced fragmentation along these lines. These $\beta$-elimination reactions most certainly are dependent on the duration of exposure to base in the coupling reactions. We have observed evidence of both the $a$ and $b$ paths (Figure 1). We have not observed either process when using oxime ether-forming chemistry.
The short spacer peptide was fitted with a terminal aminooxy moiety by substituting the last glycine in Scheme A1 with an aminooxy-modified glycine, as shown in Scheme A2. Following the standard coupling and Fmoc deprotection procedures, 2.1 was prepared for direct oximation chemistry. To verify its synthesis, the Fmoc-protected aminooxy peptide as well as the peptide 2.1 (Fmoc-deprotected) were released from the resin and confirmed by HRMS analysis (HRMS spectrum of 2.2 provided in Appendix). No overacylation (e.g., reaction at aminooxy group) was observed. Both 2.1 and 2.2 were used in subsequent oximation chemistry (see below).
B. Synthesis of poly(ethylene glycol)-based long spacers and cypate conjugates

General considerations. As originally proposed, we selected poly(ethylene glycol) (PEG) to comprise the long spacer. PEG is resistant to enzymatic cleavage, a key design feature. Our synthetic goal was to covalently attach thiol-modified (for adsorption to NGP) PEG-based spacers to cypate. In Yr01, we developed a solution phase synthesis of our target, thiol-PEG conjugates (Scheme B1, to test lengths of PEGylated long spacers — Statement of Work, Task 2b). We adapted this route to solid phase. Our initial assumption was that PEG spacers providing ‘distance-from-NGP surface’ of up to 10 nm would be required to adequately position cypate to enhance its fluorescence emission. On synthesis and evaluation of the first prototypes, ‘PEG-27’ analog 1.4a and ‘PEG-11’ analog 1.4b, revealed that the shorter analog 1.4b appeared to better position cypate. Consequently, our subsequent focus was in the shorter range.

Synthesis (Work developed in Yr01). Cysteamine hydrochloride was trityl protected and the resultant amine 1.1 (Scheme B1) was coupled to commercial PEG reagents 1.2a (n = 27, Novabiochem), 1.2b (n = 11, Novabiochem) and 1.2c (n = 7, Fluka) using PyBop as the coupling agent. Although the conjugates were quite polar, we found that purification proceeded best under normal phase (silica gel) conditions. Standard Fmoc removal was followed by EDC-mediated coupling to cypate, giving cypate conjugates 1.3a-c. These conjugates also were purified by silica gel column chromatography. HRMS analyses were performed on the trityl-protected conjugates to avoid complications arising from disulfide formation. The trityl group was cleaved prior to studies involving loading onto NGP.

Problems with solution phase synthesis: Cypate coupling to the TrS-PEG-NH₂ reagents (step 4, Scheme B1) gave mono-coupled materials 1.3a-c as well as some bis-coupled material (i.e., both carboxylic acid sidechains of cypate react). We verified formation of a bis-coupled material during synthesis of 6b. HRMS analysis of a major side product confirmed that two PEG₁₁ chains had been attached to cypate (C₁₃₇H₁₉₀N₆O₂₉S₂ [M+2H]⁺, calcd 1225.1598, found 1225.1305). In Yr02 we changed the approach for coupling the long spacer and cypate to oximation chemistry. Consequently, we set out to prepare aminooxy-terminated PEG-based spacers [as well as the complementary cypate-bis(aldehyde)] to fulfill Task 2b.
C. Synthesis of an aminooxy-terminated amphiphilic thiols.

General considerations. The aminooxy approach for the attachment of cypate to NGPs required the synthesis of a thiol-PEG-ONH$_2$ reagent that effectively binds to NGPs and presents the aminooxy group (ONH$_2$) at the surface for chemospecific ligation to aldehyde-functionalized cypate. We deviated from preparing a 'pure-PEG' compound by designing the spacer to contain a significant hydrophobic domain proximal to the thiol moiety to facilitate assembly on the gold surface (monolayer formation). Specifically, as typified by 1.4 (Scheme C1), the general design is HS-hydrophobic-hydrophilic-ONH$_2$. We expect this diblock (hydrophobic carbon chain + hydrophilic PEG chain) motif will impart technological advantages, such as (a) improved loading — the hydrophobic thiol segment is more readily anchored in the hydrophobic portion of self-assembled structures on gold nanoparticles than corresponding hydrophilic PEG-thiol spacers; (b) ease of labeling — the hydrophilic aminooxy segment is conveniently elaborated by simple mixing with aldehyde substrates in water (oximation). Analogous diblock spacers fitted with thiol and amine or alcohol functionality have been described (Agasti, 2009; Chompoosor, 2008).

Initial Synthetic Plan. Reaction of the diiodide formed from commercial PEG reagents 1.1 and 1.2 (Scheme C1) with Boc-protected hydroxylamine gave iodo-PEG reagents 1.11 and 1.21 in good yield. We found that treatment of 1.11 with nucleophiles under basic conditions surprisingly gives a cyclized product (10 membered ring) arising from intramolecular iodide displacement by the deprotonated Boc-protected aminooxy group (presumably a consequence of metal-assisted template formation via a crown ether-like transition state). Consequently, this necessitated the addition of a second Boc protecting group. Bis-(Boc-protected) analog 1.3 was prepared (Yr02) and reacted with the alcoholate derived from hydrophobic alcohol 1.4. Reagent 1.4 was prepared as described in the literature (Hornillos, 2006). Under no conditions attempted for coupling 1.3 with 1.4 was the desired adduct obtained, only Boc transfer adducts. Thus, we revised the synthetic approach to prepare the aminooxy thiol diblock spacers.

Revised Synthesis (Yr02). Our new strategy for synthesis of the aminooxy diblock spacers is shown in Figure C1. Examination of the literature for syntheses of alcohol or amineterminated diblock compounds revealed that HO-PEG-OH alkylation followed by an alkene to thiol transformation via radical methods was a viable but unwieldy route. We opted instead to alkylate HO-PEG-OH with a trityl-protected alkyl halide to obtain the aminooxy terminated target spacers. Development of such a route would constitute a new, flexible path to this useful class of compounds. Our efforts to prepare a panel of hydrophobic-hydrophilic spacers fitted with thiol and aminooxy functionality using this strategy are summarized in Scheme C2.
The bromo-alcohols 2.1a and 2.1b (Scheme C2) were transformed into the corresponding trityl-protected iodides or bromides 2.3 using standard procedures. The coupling of the hydrocarbon and PEG fragments required considerable optimization in that treatment of either class of halide with various derived alkoxides led competitively to elimination products. Ultimately, a prolonged reaction time in DMF at room temperature was discovered to afford the best overall coupling yields. In this manner, we prepared the panel of trityl-protected HS-C_12,16-PEG_4,6,8-ONH_2 compounds as depicted in Scheme C2. The aminooxy moiety was installed using Mitsunobu chemistry by reaction of the corresponding alcohols with N-hydroxyphthalimide. Hydrazinolysis smoothly afforded the free aminooxy compounds, and TFA mediated deprotection to give the thiol-aminooxy diblock spacers. These compounds were provided to our collaborator for evaluation in long spacer fluorescent enhancement studies.

Using this methodology, we also prepared a diblock TrS-C_16-PEG_8-NH_2 (16/8 amine, Scheme C2) for coupling to cypate via amide bond formation as opposed to oximation. Our synthetic route is thus readily adaptable to a variety of hydrocarbon chain lengths as well as PEG lengths and can accommodate other terminal functionalizations. We aim to publish

Scheme C2. Synthesis of Heterobifunctional Diblock Spacers. *Reagents and conditions: a. Ph_3CSH, K_2CO_3, MeOH, rt; b. I_2 (1.5 eq), PPh_3 (1.5 eq), imidazole (2 eq), THF, rt, 12 h; c. CBr_4, PPh_3, DCM d. HO-PEG_4-OH or HO-PEG_6-OH (6 eq), NaH (12 eq), DMF, rt, 48 h; e. N-hydroxyphthalimide (1.1 eq), PPh_3 (1.1 eq), DIAD (1.1 eq), THF, 0 °C – rt, 12 h; f. H_2NNH_2 (4 eq), EtOH; g. 1:1 TFA:CH_2Cl_2, Et_3SiH, rt.
these results as part of a larger study involving the optical properties of the panel’s cypate conjugates (determined by the Kang group). Representative experimental procedures and spectral characterization data for this class of compounds are provided in the Appendix.

In regard to reactivity of the diblock spacer functional groups, the aminoxy group reacts readily with aldehydes (see later subsection) as well as ketones (e.g., acetone) and, over time, the thiol group forms disulfides on exposure to oxygen. These impurities are readily separated by column chromatography (Figure C2).

Figure C2. Comparison of the $^1$H NMR spectra for the representative thiol-aminooxy diblock spacer 12/6 (black), its corresponding acetone adduct (red), and its disulfide dimer (blue).

D. Synthesis of a Cypate-bis(aldehyde).

To enable the direct labeling of NGP–ONH$_2$ (e.g., NGP treated with thiol-aminooxy diblock spacers = long spacers) with cypate using the oximation strategy, we required cypate fitted with aldehyde functionality. Our initial efforts (Yr01) to prepare either (aldehyde)-cypate-(carboxylic acid) or (aldehyde)$_2$-cypate focused on direct transformation of the carboxylic acid groups of cypate to the corresponding mono- or bis-aldehyde using the following approaches:

1. $-\text{CO}_2\text{H} \rightarrow -\text{CH}_2\text{OH}$, then selective oxidation to $-\text{CHO}$. Result: all reduction attempts (BH$_3$, THF) failed, no cypate diol isolated;
2. $-\text{CO}_2\text{H} \rightarrow -\text{C(O)Cl}$, then selective reduction to $-\text{CHO}$. Result: acid chloride successfully prepared (oxalyl chloride); however, all attempts to reduce the derived acid chloride (LiAlH(O-t-Bu)$_3$, Dibal-H) failed.

We next turned our attention to modifying cypate via bis-amide formation using a reagent that contains masked aldehyde functionality (Yr02). Using 1-ethyl-3-(3-dimethylaminopropyl) carbo-diimide (EDC) to mediate the dehydration (HOBt, with or without DIPEA, CH$_2$Cl$_2$, 0 °C to rt), cypate was successfully coupled to the amino acetal H$_2$N(CH$_2$)$_3$CH(OEt)$_2$ (Aldrich) to afford cypate amide-acetal D1.1 (Scheme D1). NOTE: the best coupling yields were obtained in the absence of base (see experimental procedure in Appendix). Amide-acetal D1.1
was characterized by $^1$H NMR and HRMS (C$_{57}$H$_{75}$N$_4$O$_6^+$ calcd 911.5681, found 911.5681).

Treatment of D1.1 with aq. acetic acid at room temperature effected hydrolysis of the acetal moieties to give the corresponding bis-aldehyde analog D1.2.

We envisioned that suitably aminooxy-functionalized nanogold particles could be directly reacted in water with bis-aldehyde derivative D1.2 to result in cybate attachment via oxime ether formation. Our collaborator confirmed this chemistry using the linkers prepared in Scheme C2 (see Kang Final Report). Note that this strategy for attaching cybate onto gold nanoparticles avoids the use of base.

To examine the reactivity of bis(aldehyde) D1.2 with our thiol-aminooxy diblock spacers, we tested the key oximation reaction independently of any nanogold particles. D1.2 was treated with spacer molecule HS-12/8-ONH$_2$ (Scheme D2) using an excess of cybate bis(aldehyde) to favor mono-oximation. Simple stirring in solution resulted in isolation (52% yield unopt.) after SiO$_2$ chromatography (HRMS given in Appendix).

**Scheme D2.** Mono-oximation of cybate-bis(aldehyde).

oximation, and the mono-product D2.1 was isolated (52% yield unopt.) after SiO$_2$ chromatography (HRMS given in Appendix).

**Polar analogs – development of a blocking group (BLK).** Initial evaluation of the heterobifunctional aminooxy spacers by the Kang lab revealed that cybate bis(aldehyde) D1.2 reacted with the spacers and became attached to NGPs; however, fluorescence emission was quenched. This suggested a closer proximity of the cybate fluorophore to the NGP surface than what we predicted based on spacer lengths. We surmised the hydrocarbon cybate moiety inserted into the C$_{11}$-hydrophobic layer surrounding the NGPs (Figure D1). One solution to address this problem was to use the remaining unreacted aldehyde group of the cybate-spacer conjugate as a means of attaching a charged/polar group to discourage the fluorophore from inserting within the hydrophobic domain. We prepared a polar ‘blocking’ group (BLK) for this purpose.

**Figure D1.** Insertion of cybate into the hydrophobic layer

Aminoalcohol D3.1 (Aldrich) was transformed into BLK using the three step sequence outlined in Scheme D3 (Biswas, 2010). Mitsunobu reaction (step a, NHP, PPh$_3$, DEAD) of $N$-hydroxyphthalimide with D3.1 followed by amine quaternization (Mel, step b) gave D3.2 in good overall yield. The phthalimide protecting group was cleaved via hydrazinolysis to give the ammonium aminooxy blocking group D3.4 in 59% overall yield (3 steps). Treatment of the cybate-spacer conjugates with D3.4 resulted in oximation of the unreacted cybate aldehyde to yield a significantly more polar conjugate. Consequently, insertion into the hydrophobic layer
was discouraged as evidenced by the restoration of fluorescence when attached to NGPs (see Kang Final Report).

It is important to note that the above described fluorescence quenching problem is not expected to be a concern for the target dual-chain cypate compound (i.e., peptide-cypate-PEG probe). Enzymatic cleavage of the peptide-cypate-PEG conjugate at its uPA recognition site affords a cypate product in which a peptide fragment (containing the polar Arg) is still attached. Consequently, the Arg is expected to function similarly to the BLK group and help discourage cypate from inserting into the hydrophobic layer.

E. Synthesis of a Tamoxifen-aldehyde.

Our experience with tamoxifen analogs (Lashley, 2000 & 2002) has allowed us to rapidly prepare a prototype tamoxifen-aldehyde for conjugation to the aminooxy thiol spacers (thus, for attachment to NGPs). Analog E1.1 (Scheme E1) is a silyl-protected archival intermediate available from a project in our laboratories (Lashley 2002). As part of Task 2d — conjugate tamoxifen onto NGPs — we re-examined its conversion into a suitable aldehyde for use in our oximation approach to functionalizing NGPs (unpublished). Fluoride-mediated desilylation (step a, Scheme E1) of E1.1 (9 mg) followed by Dess-Martin oxidation (step b) gave aldehyde intermediate E1.2. Hydrolysis of the MOM protecting group (step c: THF, 1N HCl, 75 °C, 3h) afforded aldehyde E1.3 (3 mg provided to our collaborator; see Appendix for experimental data).

F. Synthesis of the dual-chain target HS-peptide-cypate-PEG-SH

We have explored three approaches to prepare a dual-chain cypate conjugate containing both a ‘short chain’ (peptide) and a ‘long chain’ (PEG) as proposed.

1. Solid-phase amide coupling approach. Using the chemistry developed in Scheme A1, we attached cypate to the resin-supported peptide %-GGRGGS-NH₂ via amide coupling conditions. The bound cypate (free -CO₂H group) was then treated with trityl-protected amine spacer TrS-C₁₈PEG₉-NH₂ (prepared according to Scheme C2) under amide-forming conditions. Two separate attempts were made: first attempt — DIC, HOBt, DIPEA; second attempt — EDC,
HOBt. Cleavage of the material from the resin of both coupling experiments using the established conditions (TFA, TES, CH₂Cl₂) gave a mixture of products in each case, with a primary component identified by HRMS as the β-elimination product (see Figure A1) depicted below. A variety of MS techniques were examined including MALDI, but we did not observe any evidence for the formation of the expected dual chain adduct.

![Acrylamide derivative formed on β-elimination](image)

2. **Solid-phase oximation coupling approach.** Using our aminooxy coupling approach, we attached the cypate bis(aldehyde) D1.2 (Scheme D1) to the resin-supported aminooxy peptide 2.1 (Scheme A2) by stirring in DMF at room temperature overnight. Attachment of cypate in this manner was visually evident by the change in color of the resin after reaction with the bis(aldehyde) analog (beads changed from yellow to blue, analogous to the change in the amide-coupling approach). We then reacted the cypate-loaded resin with aminooxy spacer TrS-C₁₂PEG₈-ONH₂. Cleavage from the resin was conducted using TFA and Et₃SiH. Analysis of the products revealed that the N-O bond was cleaved under these conditions (likely due to reduction by the Et₃SiH reagent). HRMS identification of the hydroxy peptide shown below indicates that the RO-N=C linkage (oxime ether) does not survive the cleavage conditions. It is noteworthy that the less basic RO-NH₂ linkage does survive these conditions (see Scheme A2, 2.1→2.2). Consequently, the cleavage reaction was attempted a second time using only TFA (no reducing agent). Not unexpectedly, these conditions afforded a product mixture from which we were unable to isolate the desired dual-chain product.

![Hydroxyl derivative formed on RO-N reduction](image)

3. **Solution-phase oximation coupling approach.** We reacted aminooxy spacer HS-C₁₂PEG₈-ONH₂ with cypate bis(aldehyde) D1.2 in CH₂Cl₂:MeOH (9:1) according to Scheme D2 to form mono-adduct D2.1. The adduct was purified and confirmed by HRMS prior to reaction with aminooxy peptide 2.2 (Scheme A2) in CH₂Cl₂:MeOH (1:1). The desired dual-chain conjugate (shown on the next page) was formed in the reaction as evidenced by HRMS analysis of the crude product: HS-C₁₂PEG₈-ON=C-Cypate-C=NO-GGRRGG-C(O)NHCH₂CH₂SH, C₉₅H₁₄₅N₁₅O₁₈S₂⁺ [M⁺ + H⁺]/2 calcd 924.0164, found 924.0161 (m/z where z = 2, see Appendix for spectrum).

Unfortunately, we have been unable to remove undesired by-products due to the sensitivity of cypate and its conjugates toward SiO₂ chromatography (induced β-eliminations?). We also explored this solution phase oximation approach by altering the order of addition (e.g., aminooxy peptide added to cypate, then addition of aminooxy spacer), but did not improve our ability to isolate pure dual-chain adduct.
Target prototype dual-chain cypate conjugate

**Recommendations for future attempts.** The aminooxy approach showed the greatest promise for synthesis of this challenging molecule. We recommend exploring a solid phase route since the advantages for good initial mono-functionalization (due to polymer site isolation) and product purification are substantial. Formation of bis-derivatives is a problem when using solution-phase approaches. The problems we encountered are related to the sensitivity of cypate to base and to purification via chromatography, and the sensitivity of the N-O linkage to the resin cleavage conditions. The use of mild base-free conditions, such as oximation, and acid- and reductant-free cleavage conditions will greatly improve the chances for a successful synthesis.

Another attractive option, one that mimics a solid-phase synthesis, is to assemble the cypate conjugate in situ on the NGPs. This approach would entail loading cypate bis(aldehyde) via simple mixing (oximation) onto NGP-peptide-aminooxy—much like loading onto resin—followed by addition of the thiol-aminooxy spacer. The hydrophobic domain of the spacer molecule will insert and load onto the NGPs, and the terminal aminooxy groups will couple with the free aldehyde group of the conjugated cypate, thus completing an in situ synthesis. We recommend that this approach be tested and are providing our collaborators with the three components needed to explore this option.

Finally, a refocusing of the project, given the lessons learned from the past two years, is another option, and this would suggest targeting a single-chain probe in which the PEG chain loops from a substituent group off of the peptide backbone and attaches to cypate’s second carboxyl (or aldehyde) group. Furthermore, there are structural changes that can be made to cypate to minimize its propensity for elimination reactions, such as the inclusion of an additional methylene to the acrylic acid sidechains.
Key Research Accomplishments

• Secured syntheses of peptide–cypate conjugates fitted with thiol functionality
  Key observation (from partnering award W81XWH-08-1-0460): the overall length of the peptide spacer in peptide-cypate conjugate 1.3 (Scheme A1) is suitable in that 81-86% of cypate fluorescence is quenched relative to controls when loaded onto 5 nm gold particles. Importantly, the peptide sequence has been shown by our collaborator to undergo cleavage on treatment with the enzyme uPA.

• Secured syntheses of PEG–cypate conjugates fitted with thiol functionality
  Key observation (from partnering award W81XWH-08-1-0460): fluorescence enhancement greater than 10% was noted, although not optimized, only at lengths shorter than the overall length of the HS-PEG27-cypate conjugate, calculated to be ~10 nm. This obviates syntheses of longer spacers for attachment to nanogold particles ≤ 10 nm in size.

• Developed a new synthesis of thiol-diblock-aminooxy spacers of the type HS-CnPEGm-ONH2
  A 5-step sequence from bromo alcohols to the heterobifunctional spacers was developed. The route can accommodate variable hydrophobic and hydrophilic domain lengths as well as different terminal functionality. These spacers readily react with aldehydes via oximation and attach to NGPs via the thiol moiety, both by straightforward mixing operations.

• Prepared a novel cypate–bis(aldehyde) substrate for use in conjugation (oximation) reactions
  The availability of compound D1.2 has facilitated the attachment of cypate to aminooxypeptide- and aminooxyPEG-based spacers positioned on nanogold particles. The oximation strategy using D1.2 has been shown by us and our collaborators to proceed nicely to form oxime ether conjugates of the sensitive cypate probe.

• Discovered that cypate polarity must be modulated to avoid fluorescence quenching
  We and our collaborators showed that the attachment of a blocking group (BLK, Scheme D3) restored the fluorescence emission of a diblock-cypate conjugate on NGPs. This work demonstrated indirectly that cypate may prefer hydrophobic environments, and if such an environment is too near the NGP surface, then quenching may occur. The attachment of the BLK group altered the polarity such that interaction with the hydrophobic environment was discouraged.

• Identified paths whereby cypate and its derivatives degrade
  We have elucidated a major decomposition pathway of cypate and its derivatives (Figure A1). Apparently, β-elimination occurs when cypate analogs are exposed to base. For this reason, cypate analog generation was optimized using base-free oximation chemistry.

Key research accomplishments related to the optical properties of materials prepared during the course of this project will be described in our collaborator’s Final Report (W81XWH-08-1-0460)
Reportable Outcomes

This award is partnered with award W81XWH-08-1-0460 (PI: Kang). The co-deliverables are listed below (documents not attached as part of this report).

- **Manuscript**
  

- **Conference Abstracts**
  


- **United States Patent Application**
  

  **STATEMENT OF GOVERNMENT INTEREST.** This invention was made with government support under DOD Grant No. W81XWH-08-1-0460 awarded by Department of Defense (DOD). The government has certain rights in the invention.

  **FIELD OF THE INVENTION.** A field of the invention is in vivo imaging and cell detection via fluorescent markers. Example applications of the invention include fluorescence detection of breast cancer and other cancers with a site specific enhanced fluorescence marker.

- **Doctor of Philosophy Research**
  
  This award has provided funds to train Mr. Souvik Biswas and Mr. Sebastien Laulhe as a Graduate Student Researchers during their third year of graduate school (UofL, Ph.D. program in Chemistry).

- **Honors Thesis**
  
  Mr. Joseph D. Moore (UofL undergraduate student) conducted Honors Thesis research during the Summer and Fall of 2009 using funds from this project. His thesis entitled “Thiol Aminooxy Diblock Spacers: Synthesis and Application in Fluorophore-Nanogold Cancer Detection Probes” received the College of Arts & Science’s Best Honors Thesis Award in March 2010.
Summary of chemistry personnel receiving pay from the research effort:

Dr. Michael H. Nantz (PI – Yr01-2)
Mr. Souvik Biswas (Graduate student – Yr01)
Mr. Sebastien Laulhe (GradYr02)
Mr. Joseph D. Moore (Summer beginning of Yr02)

Additional personnel involved with the project but not funded by the research effort:

Dr. Archna Massey
Ms. Stephanie Mattingly

Conclusion

We have developed the synthetic routes necessary to prepare thiol terminated peptide- and PEG-based conjugates of cypate, an indocyanine green (FDA-approved) derivative with two pendant carboxylic acid groups developed by our collaborator Dr. S. Achilefu. Examples of both types of derivatives have been prepared and then evaluated by our collaborator, Dr. K. Kang, as part of the joint effort to optimize the fluorescence properties of the proposed nano-entity as well as to study the uPA cleavage properties of the peptide-cypate conjugate.

A key aspect of the conjugation research involved attaching peptide- or PEG-based spacers to the cypate carboxylic acid groups via amide bond formation. We have also developed a milder method of attaching either peptide or PEG-based spacers to cypate, namely oximation coupling. As part of this research, we secured a new synthesis of heterobifunctional thiol-aminooxy hydrophobic-hydrophilic diblock spacers of the type HS-C\textsubscript{n}H\textsubscript{2n-PEG\textsubscript{m}}ON\textsubscript{2}. A cypate bis(aldehyde) substrate was prepared to complement these aminooxy spacers to exploit the chemospecific oximation chemistry. Simple mixing of the cypate bis(aldehyde) and aminooxy spacers, as well as aminooxy-functionalized peptides, results in the desired conjugation chemistry via oxime ether linkage formation. We also prepared a tamoxifen aldehyde for mild labeling of NGPs to exploit the oximation strategy.

Our attempts to prepare the proposed dual-chain conjugate of cypate have shown promise (a bis(oxime ether) version of the prototype has been prepared), but efforts to obtain the conjugate in pure form have failed. We have identified some of the paths whereby the sensitive cypate fluorophore gives undesired by-products, and these observations led us to focus on the oximation path (base-free). In situ assembly on NGPs, using the appropriate aminooxy (peptide and PEG spacers) and aldehyde (cypate) substrates, is likely the best approach to examine as a next step.
References


Appendices

A. Experimental Procedures

1. General

Solid phase peptide synthesis was performed using methoxy-activated, cysteamine-loaded polystyrene resin (Novabiochem, cat. no. 01-64-0086). All amino acids (Novabiochem) and peptide coupling reagents (Aldrich Chemical Company) were used as received from vendors. DMF (Aldrich) was used as received, and CH$_3$CN (Pharmaco) was filtered and degassed (N$_2$) prior to use. HPLC analyses and purifications were conducted on a Waters Corporation Delta 600 instrument. High resolution mass spectrometry services were provided by the Center for Regulatory and Environmental Analytical Metabolomics at the University of Louisville; FTCIR measurements were taken on a Thermo LTQ-FT instrument (7 tesla). Cypate was provided by our collaborator Dr. Samuel Achilefu (Washington University, St. Louis).

2. Solid phase synthesis of conjugate 1.3 (steps according to Scheme A1)

   Steps 1&2. Cysteamine-loaded polystyrene resin (200 mg, 0.14 mmol active amine) was treated with Fmoc-protected diglycine (Novabiochem, 150 mg, 0.42 mmol) in the presence of diisopropylcarbodiimide (DIC, 0.064 mL, 0.42 mmol), N-hydroxybenzotriazole (HOBt, 56 mg, 0.42 mmol) and N,N-diisopropylethylamine (DIPEA, 0.12 mL, 0.84 mmol) in DMF (3 mL). After shaking overnight, the resin was filtered and thoroughly washed with CH$_2$Cl$_2$ (7X). The resin then was dried under vacuum, swelled using DMF, filtered, and treated with 20% piperidine in DMF (3 mL) at room temperature for 40 mins. The piperidine washing process was repeated once. The resin was filtered and washed successively with DMF (3X) and CH$_2$Cl$_2$ (5X), and then dried under vacuum.

   Steps 3&4. The diglycine loaded resin (®-G-G-NH$_2$) was swelled using DMF over 30 mins, filtered, and then treated with Fmoc-protected arginine-Boc$_2$ (Novabiochem, 250 mg, 0.42 mmol), DIC (0.064 mL, 0.42 mmol), HOBt (56 mg, 0.42 mmol) and DIPEA (0.12 mL, 0.84 mmol) in DMF (3 mL). After shaking overnight, the resin was filtered and thoroughly washed with CH$_2$Cl$_2$ (7X). The resin was dried under vacuum, swelled using DMF, filtered, and then treated with 20% piperidine in DMF (3 mL) at room temperature for 40 mins. The piperidine washing process was repeated once. The resin was filtered and washed successively with DMF (3X) and CH$_2$Cl$_2$ (5X), and then dried under vacuum.

   Steps 5&6. The ®-G-G-R-NH$_2$ resin was swelled using DMF over 30 mins, filtered, and then treated with Fmoc-protected diglycine (Novabiochem, 250 mg, 0.70 mmol), DIC (0.108 mL, 0.70 mmol), HOBt (90 mg, 0.70 mmol) and DIPEA (0.25 mL, 1.4 mmol) in DMF (3 mL). After shaking overnight, the resin was filtered and thoroughly washed with CH$_2$Cl$_2$ (7X). The resin was dried under vacuum, swelled using DMF, filtered, and then treated with 20% piperidine in DMF (3 mL) at room temperature for 40 mins. The piperidine washing process was repeated once. The resin was filtered and washed successively with DMF (3X) and CH$_2$Cl$_2$ (5X), and then dried under vacuum.

   Steps 7&8. The ®-G-G-R-G-NH$_2$ resin was swelled using DMF over 30 mins, filtered, and then treated with Fmoc-protected glycine (Novabiochem, 205 mg, 0.70 mmol), DIC (0.108 mL, 0.70 mmol), HOBt (90 mg, 0.70 mmol) and DIPEA (0.25 mL, 1.4 mmol) in DMF (3 mL). After shaking overnight, the resin was filtered and thoroughly washed with CH$_2$Cl$_2$ (7X). The resin was dried under vacuum, swelled using DMF, filtered, and then treated with 20% piperidine in DMF (3 mL) at room temperature for 40 mins. The piperidine washing process was repeated once. The resin was filtered and washed successively with DMF (3X) and CH$_2$Cl$_2$ (5X), and then dried under vacuum.
To confirm peptide integrity prior to coupling with cypate, a small portion of the peptide was cleaved from the resin by shaking the ®-G-G-R-G-G-NH₂ resin in a 1:10:10 mixture of triethylsilane:TFA:CH₂Cl₂ at room temperature for 4h. The reaction was filtered and solvents removed under reduced pressure. The residue was washed with Et₂O (7X) and then dried under vacuum. Analysis of the crude cysteamine-hexapeptide conjugate by HPLC (Atlantis dC18 5 mm column, gradient elution using 5% CH₃CN/H₂O to 95% CH₃CN/H₂O over 30 min) indicated excellent sample homogeneity (retention time = 25.6 min); HRMS (FTCIR): C₃₃H₄₅N₁₀O₈S [M+H]⁺, calcd 741.3137, found 741.3133.

Step 9. To the ®-G-G-R-G-G-NH₂ resin (65 mg, theoretical 0.045 mmol peptide) in DMF at room temperature was added cypate (140 mg, 0.227 mmol), 3-(diethoxyphosphoryloxy)-1, 2, 3-benzotriazin-4(3H)-one (DEPBT, Aldrich, 68 mg, 0.227 mmol), HOBt (30 mg, 0.227 mmol), and DIPEA (0.08 mL, 0.45 mmol). After shaking overnight, the resin was filtered and thoroughly washed with CH₂Cl₂ (7X). The resin then was dried under vacuum.

Step 10. To the ®-G-G-R-G-G-NHC(O)-cypate resin prepared above (15 mg, theoretical 0.01 mmol) in CH₂Cl₂ (0.3 mL) at room temperature was added triethylsilane (0.06 mL). After shaking 15 minutes, trifluoroacetic acid (0.04 mL) was added and the resin suspension was shaken 1 h, allowed to stand 1 h, and then shaken 3 h. The resin was filtered and the filtrate was concentrated by rotary evaporation. The residue was washed with Et₂O (5X) and dried under vacuum to afford the target cysteamine-hexapeptide-cypate conjugate (7 mg, 62% based on resin loading, 12% based on cypate consumption); HRMS (FTCIR): C₅₉H₇₃N₁₂O₉S [M]⁺, calcd 1125.5338, found 1053.5114 (loss of acrylic acid [M⁺–HO₂CCH=CH₂] as shown below, calcd for C₅₆H₆₉N₁₂O₇S, 1053.5127, see Appendix B2 for spectrum).


Preparation of the short linker peptide terminated with an aminooxy moiety was performed using solid phase synthesis. The synthesis follows the standard procedure for solid phase synthesis of the amino-terminated short linker peptide outlined in Scheme A1 to the stage of step 7. Addition of HOOCCH₂ONHfomic to the resin-supported ®-GGRGG-G-NH₂ fragment is performed as follows (steps below refer to chemistry depicted in Scheme A2):

Steps 1&2 (SL2-74): The ®-G-G-R-G-G-NH₂ resin (100 mg) was swelled using DMF over 30 mins, filtered, and then treated with Fmoc-protected aminooxyglycine (Polypeptide, 75 mg, 0.21 mmol), DIC (0.033 mL, 0.21 mmol), HOBt (28 mg, 0.21 mmol) and DIPEA (0.075 mL, 0.42 mmol) in DMF (2 mL). After shaking overnight, the resin was filtered and thoroughly washed with CH₂Cl₂ (7X). The resin was dried under vacuum, swelled using DMF, filtered, and then treated with 20% piperidine in DMF (3 mL) at room temperature for 40 mins. The piperidine washing process was repeated once. The resin was filtered and washed successively with DMF (3X) and CH₂Cl₂ (5X), and then dried under vacuum.
To confirm the integrity of the aminooxy peptide linker, a sample of the resin was treated with TFA (50%), triethylsilane (5%) in CH₂Cl₂ at room temperature to cleave the peptide from the resin as well as to deprotect the arginine. Analysis by HRMS confirmed the title compound; HRMS, C₁₈H₃₄N₁₀O₇S, calcd 534.2411, found 535.2411 (see Appendix for spectrum).

4. Solution phase synthesis of TrS-PEG-cypate 1.3b (steps according to Scheme B1)

Step 2. To PEG-reagent 1.2b (110 mg, 0.13 mmol) and trityl-protected cysteamine 1.1 (42 mg, 0.13 mmol) in CH₂Cl₂ (5 mL) at room temperature was added HOBt (18 mg, 0.13 mmol) and DMF (0.2 mL). The reaction mixture was cooled to 0 °C and DIPEA (0.56 mL, 0.33 mmol) was added followed by addition of a solution of PyBop (68 mg, 0.13 mmol) in CH₂Cl₂ (2 mL). The mixture was gradually warmed to room temperature and stirred overnight. The reaction solvents were removed under high vacuum. The solid residue was dissolved in EtOAc and the solution was washed successively with water, then brine and subsequently dried (Na₂SO₄). After filtration, the solvent was removed by rotary evaporation and the crude product purified by column chromatography (SiO₂, 2% MeOH in CH₂Cl₂) to afford the amide product (109 mg).

Step 3. The TrS-PEG-NHFmoc product (104 mg, 0.091 mmol) was treated with piperidine (0.168 mL) in DMF (2 mL) at room temperature. After stirring 4h, the DMF was removed under high vacuum. The residue was washed with Et₂O (5X) and dried under vacuum to give the deprotected product (63 mg, 79%). HRMS, calc’d C₉₈H₁₄₀N₁₆Na₂S 919.4984, found 919.4995.

Step 4. To a suspension of the TrS-PEG-NH₂ product (52 mg, 0.056 mmol), HOBt (8 mg, 0.059 mmol) and cypate (35 mg, 0.056 mmol) in CH₂Cl₂ (3 mL) at room temperature was added DMF (0.2 mL). The reaction mixture was cooled to 0 °C and DIPEA (22 mg, 0.17 mmol) was added followed by addition of a solution mixture of EDC (9 mg, 0.47 mmol) in CH₂Cl₂ and DMF. After stirring in the dark at room temperature overnight, the reaction was quenched by addition of water and extracted using CH₂Cl₂. The organic layer was washed with brine and then dried (Na₂SO₄). The solvents were removed by rotary evaporation and the residue purified by column chromatography (SiO₂, gradient elution 9-15% MeOH in CH₂Cl₂) to obtain mono-coupled product 1.3b (7 mg, 9%) [HRMS, C₈₉H₁₁₃N₄NaO₁₆S [M+Na⁺], calcd 1548.7770, found 1543.8318] and bis-coupled product (12 mg, 10%) [HRMS, C₁₅₇H₁₉₀N₁₆O₂₅S₂ [M+2H⁺], calcd 1225.1598, found 1225.1305].

5. Synthesis of heterobifunctional PEG spacer 1.3 (steps according to Scheme C1)

Step 1. 16.02 g of NaI (106.9 mmol), 10.0 g (53.45 mmol) of 1,2-bis(2-chloro-ethoxy)ethane were dissolved in 110 mL of acetone and fitted the flask with a reflux condensor. The reaction mixture was refluxed for 20 h, cooled to room temperature, removed the acetone by vacuum, and added 150 mL of chloroform. Filtered the precipitate using frit funnel, removed the filtrate and bis(2-chloro-ethoxy)ethane 1.1 as an oil; ¹H NMR (CDCl₃) δ 7.57 (br s, 1H), 4.06-4.0 (m, 2H), 3.80-3.70 (m, 4H), 3.69-3.61 (m, 4H), 3.27 (t, J = 7 Hz, 2H), 1.48 (s, 9H).
Step 3. To a solution of 1.11 (200 mg, 0.53 mmol) in acetonitrile (6 mL) at room temperature was added (Boc)₂O (175 mg, 0.79 mmol). The reaction mixture was cooled to 0 °C, added 65 mg (0.53 mmol) of DMAP and stirred at room temperature for 24 h by monitoring the starting material consumption using TLC. Added 20 mL of water to quench the reaction followed by 50 mL saturated NaHCO₃ solution. Extracted with 300 mL of ethylacetate and washed with 1N HCl (50 mL), 5% KHSO₄ (50 mL), and finally with 50 mL of brine solution. The organic layer was concentrated under vacuum and purified by column chromatography. Yield: 200 mg (79%). ¹H NMR (CDCl₃) δ 4.10 (t, J = 4.5 Hz, 2H), 3.75 (t, J = 6 Hz, 4H), 3.71 - 3.65 (m, 4H), 3.26 (t, J = 7 Hz, 2H), 1.54 (s, 18H); ¹³C NMR (CDCl₃) δ 150.0, 83.7, 75.4, 72.0, 70.5, 70.1, 68.6, 28.0, 2.8.

6. Representative Experimental Procedures for Synthesis of Thiol Aminooxy Spacers (Scheme C2)

TrS(CH₂)₁₂OH. To a solution of 12-bromo-dodecan-1-ol (1.0 g, 3.78 mmol) in MeOH (40 mL) at room temperature was added potassium carbonate (1.04 g, 7.56 mmol). After stirring 5 minutes, triphenylmethanethiol (1.36 g, 4.90 mmol) was added, and the reaction was stirred at room temperature for 16 hours. The reaction solution was then diluted with diethyl ether (100 mL), transferred to a separatory funnel, and neutralized with a Na₂HPO₄/NaH₂PO₄ buffer (10 mL). The buffer was removed, and the organic layer was then washed with NaCl brine (3 × 75 mL). The aqueous layers were combined and extracted with diethyl ether (25 mL), and the organic layers were combined, dried over Na₂SO₄ and concentrated by rotary evaporation. The crude residue was purified by flash chromatography (12 g SiO₂; CH₂Cl₂:MeOH (v:v) 100:0 → 95:5) to afford the desired product as a clear oil (1.71 g, 3.70 mmol, 99% yield). ¹H NMR (500 MHZ, CDCl₃): 7.34 (d, J=7.5 Hz, 6H), 7.20 (t, J=7.5 Hz, 6H), 7.13 (t, J=7.4 Hz, 3H), 3.56 (t, J=4.5 Hz, 2H), 2.06 (t, J=7.5 Hz, 2H), 1.49 (qn, J=7 Hz, 2H), 1.02-1.35 (m, 4H), 1.27-1.38 (m, 14H).

TrS(CH₂)₁₂Br. To an evacuated and N₂ purged flask containing TrS(CH₂)₁₂OH (0.900 g, 1.95 mmol), sodium bicarbonate (0.164 g, 1.95 mmol), and a stir bar, dry CH₂Cl₂ (30 mL) was added by syringe. In separate flasks, carbon tetrabromide (1.618 g, 4.88 mmol) and triphenylphosphine (1.330 g, 5.07 mmol) were each purged with N₂, and minimal dry CH₂Cl₂ was added by syringe to dissolve each solid. Both the carbon tetrabromide and triphenylphosphine were cannulated into the reaction flask, and the solution was stirred for 12 hours. The resulting dark red solution was diluted with Et₂O and washed with NaCl brine (3 × 50 mL). The aqueous layers were combined and extracted with Et₂O (50 mL), and all organic extracts were combined and washed once more with NaCl brine (100 mL). The organic solution was dried over Na₂SO₄, concentrated by rotary evaporation, and purified by flash column chromatography (12 g SiO₂; CH₂Cl₂, 100 mL) to give the desired product (0.989 g, 1.89 mmol, 96% yield) as a white solid. ¹H NMR (500 MHZ, CDCI₃): 7.34 (d, J=7.5 Hz, 6H), 7.20 (t, J=7.5 Hz, 6H), 7.13 (t, J=7.4 Hz, 3H), 3.56 (t, J=4.5 Hz, 2H), 2.06 (t, J=7.5 Hz, 2H), 1.49 (qn, J=7 Hz, 2H), 1.02-1.35 (m, 17H).

TrS(CH₂)₁₂OPEG₈OH. To a suspension of sodium hydriode (0.292 g, 12.17 mmol) in DMF (10 mL) at 0°C under N₂, a solution of vacuum-dried tetra(ethylene glycol) (1.201 g, 6.18 mmol) in anhydrous DMF (20 mL) under N₂ was transferred by cannula, and the reaction flask was purged with N₂. After 5 minutes, the solution turned a pale yellow color. Then TrS(CH₂)₁₂Br (0.54 g, 1.03 mmol) in anhydrous DMF (25 mL) under N₂ was transferred to the reaction flask in the same manner. The reaction was allowed to come to room temperature, kept under N₂ atmosphere, and stirred for 72 hours. The resulting dark yellow solution was then dissolved in Et₂O (75 mL), transferred to a separatory funnel, and washed with NaCl brine (75 mL) mixed
with RO water (5 mL). Aqueous layers were combined and extracted with Et2O (50 mL), and then organic layers were combined and washed once more with NaCl brine (50 mL). The final organic extract was then dried over Na2SO4 and concentrated by rotary evaporation. The crude residue was purified by flash column chromatography (12 g SiO2; CH2Cl2:MeOH (v:v) 100:0 → 90:10) to give the desired product as a yellow oil (0.268 g, 0.422 mmol, 41% yield). 1H NMR (500 MHz, CDCl3): δ = 4.5 Hz, 2H), 3.87 (t, J=4.5 Hz, 2H), 3.67 (t,

TrS(CH2)12OPEG8ONPh. To a solution of product TrS(CH2)12OPEG8OH (0.220 g, 0.345 mmol) in dry THF (5 mL) under N2 at 0°C were added N-hydroxysphthalimide (0.068 g, 0.414 mmol) and triphenylphosphine (0.109 g, 0.414 mmol). Over the next 5-10 minutes, diisopropyl azodicarboxylate (0.082 mL, 0.414 mmol) was added dropwise by syringe. The solution quickly turned a dark yellow-orange. The reaction flask was then allowed to return to room temperature and stirred overnight to yield an opaque golden solution. The mixture then was concentrated by rotary evaporation, diluted with EtOAc (100 mL), transferred to a separatory funnel, and washed with NaCl brine (3 × 75 mL). The organic layer was then dried over Na2SO4, concentrated by rotary evaporation, and then further purified by vacuum pump.

TrS(CH2)12OPEG8ONH2. To a solution of TrS(CH2)12OPEG8ONPh (0.131 g, 0.168 mmol) in CH2Cl2 (5 mL), hydrazine monohydrate (0.163 mL, 3.35 mmol) was added at room temperature. Within 10 seconds, the solution changed from transparent to dark red, and back to transparent. The reaction was stirred for 3 hours, and the solution changed color once again to an opaque white. The CH2Cl2 was then removed by rotary evaporation, and the resulting white solid was purified by flash chromatography (8 g SiO2; CH2Cl2:MeOH (v:v) 99:1 → 90:10) to afford the desired product as a pale yellow oil (0.106 g, 0.163 mmol, 98% yield).

HS(CH2)12OPEG8ONH2. To a N2 purged solution of TrS(CH2)12OPEG8ONH2 (0.080 g, 0.123 mmol) in CH2Cl2 (0.5 mL), trifluoroacetic acid (0.5 mL, excess), and triethylsilane (0.085 mL, 0.073 mmol) were added at room temperature. The solution immediately turned dark yellow, then clear. The reaction was kept under N2 atmosphere and stirred for 3 hours. The volatiles were then removed by rotary evaporation and subsequent vacuum pumping, and the resulting white solid was purified by flash chromatography (5 g SiO2; CH2Cl2:MeOH (v:v) 9:1) to afford the desired product as a pale yellow oil (0.035 g, 0.086 mmol, 70% yield). 1H NMR (500 MHz, CDCl3): 5.56 (s, br), 3.84 (t,
J=4.5 Hz, 2H), 3.62-3.70 (m, 12H (plus H2O impurities)), 3.58 (t, J=3 Hz, 2H), 3.44 (t, J=6.5 Hz, 2H), 2.52 (q, J=7.5 Hz, 2H), 1.57 (m, J=7 Hz, 2H), 1.23-1.41 (m, 18H); 13C NMR (125 MHz, CDCl3): δ = 71.79, 70.85, 70.81, 70.78, 70.30, 69.86, 29.79, 29.72, 29.30, 28.61, 23.14, 23.08; FTMS calcd for C20H45NO5S [M + Na] 432.2760, found 432.2760.

7. Synthesis of the amino spacer TrS-C16-PEG8-NH2 (Scheme C2)

TrS-C16-PEG8-NPhth. To a solution of TrS-C16-PEG8-OH (Scheme C2) (0.124 g, 0.142 mmol) in dry THF (5 mL) under N2 at 0°C were added Nphthalamide (0.025 g, 0.171 mmol) and triphenylphosphine (0.044 g, 0.171 mmol). Over the next 5-10 minutes, disopropyl azodicarboxylate (0.034 mL, 0.171 mmol) was added dropwise by syringe. The reaction flask was then allowed to return to room temperature and stirred overnight to yield an opaque golden solution. The mixture was then concentrated by rotary evaporation, washed with NaCl brine (3 × 75 mL), transferred to a separatory funnel, and washed with NaCl brine (3 × 75 mL). The organic layer was then allowed to return to room temperature over night. The amino acetal (EtO)2NCH(CH3)2NPhth. (0.044 g, 0.057 mmol) was added by chromatography (SiO2; Hexane:EtOAc (v:v) 90:10, followed by wash MeOH:NH4Cl:DCM (45:5:50)) to afford the desired product as a pale yellow oil (0.044 g, 0.058 mmol). The crude residue was purified by chromatography (SiO2; Hexane:EtOAc (v:v) 60:40) to give the desired product as a bright yellow oil (0.134 g, 0.134 mmol, 95% yield); 1H NMR (400 MHz, CDCl3): 7.84 (dd, 2H), 7.70 (dd, 2H), 7.40 (d, 6H), 7.27 (t, 6H), 7.20 (t, 3H), 3.90 (t, 2H), 3.73 (t, 2H), 3.55-3.64 (m, 28H), 3.44 (t, 2H), 2.13 (t, 2H), 1.56 (qn, 2H), 1.37 (qn, 2H), 1.10-1.35 (m, 24H); 13C NMR: 168.21, 145.069, 133.88, 132.13, 129.58, 127.75, 126.45, 123.20, 70.57, 70.55, 70.52, 70.06, 70.02, 67.95, 67.88, 37.23, 32.23, 30.29, 29.65, 29.61, 29.54, 29.48, 29.40, 29.16, 28.99, 28.56, 26.07, 25.58; HRMS: C59H83NO10S (M+Na), calcd 1020.5635, found 1020.5651.

TrS-C16-PEG8-NH2. To a solution of TrS-C16-PEG8-NPhth prepared above (0.070 g, 0.07 mmol) in CH2Cl2 (3 mL), hydrazine monohydrate (0.033 mL, 0.617 mmol) was added at room temperature. The reaction was stirred for 1.5 hours, the solution changed to an opaque white. The CH2Cl2 was then removed by rotary evaporation, and the resulting white solid was purified by chromatography (SiO2; CH2Cl2:MeOH (v:v) 99:1 → 90:10, followed by washing with MeOH:NH4OH:DCM (45:5:50)) to afford the desired product as a pale yellow oil (0.044 g, 0.05 mmol, 72% yield); 1H NMR (500 MHz, CDCl3): 7.40 (d, 6H), 7.27 (t, 6H), 7.20 (t, 3H), 3.92 (t, 2H), 3.75 (t, 2H), 3.55-3.67 (m, 24H), 3.57 (t, 2H), 3.44 (t, 2H), 3.17 (t, 2H), 2.13 (t, 2H), 1.56 (qn, 2H), 1.37 (qn, 2H), 1.10-1.35 (m, 24H); HRMS: C55H83NO9S, (M+H) calcd 868.5761, found 868.5773.

8. Solution phase synthesis and hydrolysis of cypate-bis(aldehyde) (Scheme D1)

Optimized procedure (SL2-87): To a mixture of cypate (75 mg, 0.12 mmol), HOBt (64 mg, 0.48 mmol), and EDC (92 mg, 0.48 mmol) in dry DMF (2 mL) at room temperature was added the amino acetal (EtO)2NCH(CH3)2NPhth (0.085 mL, 0.48 mmol). After stirring in the dark at room temperature overnight, the reaction was quenched by addition of water and extracted using CH2Cl2. The organic layer was washed with brine and then dried (Na2SO4). The solvents were removed by rotary evaporation and the residue purified by column chromatography (SiO2, eluting with a gradient of 1-7% MeOH in CH2Cl2) to obtain bis-coupled product D1.1 (74 mg, 68%); HRMS, C57H75N2O6·[M + H]+, calcd 911.5681, found 911.5665 (Appendix, Section B5); 1H NMR (400MHz, CDCl3): δ = 8.06 (m, 3H), 7.92 (m, 4H), 7.78-7.56 (m, 8H), 7.43 (tr, 2H), 7.29 (m, 2H), 6.70 (bs, 2H), 6.47 (bs, 2H), 4.50 (tr, 4H), 4.39 (s, 2H), 3.59 (qn, 4H), 3.43 (qn, 4H), 3.24 (q, 4H), 2.95 (tr, 4H), 1.92 (s, 12H), 1.58 (bs, 8H), 1.15 (tr, 12H); 13C NMR: 169.40, 139.63,
131.79, 130.67, 130.10, 128.64, 128.12, 127.46, 124.78, 121.95, 111.09, 102.69, 61.21, 50.68, 39.52, 31.15, 27.50, 24.56, 15.31.

_Hydrolysis of D1.1._ The bis-acetal was hydrolyzed by dissolving 15 (4.7 mg) in 30% aqueous acetic acid (3 mL) followed by stirring 3.5 h at room temperature. The solvents were then removed under vacuum to obtain aldehyde 16 (3.5 mg) as a green solid; HRMS, C_{49}H_{55}N_{4}O_{4}^+ [M + H]^+, calcd 763.4218, found 763.4227 (Appendix, Section B6).

9. **Synthesis of tamoxifen-aldehyde E1.3** (Scheme E1)

**Compound E1.1:** 1 mL of dry THF and TBAF (0.045 mL, 0.045 mM) was added to 1 (9 mg, 0.015 mM) at room temperature. The reaction was quenched with saturated NaHCO$_3$ after 3 h and the aqueous layer was extracted with ether (10 mL, 3X). The organic layer was washed with water (10 mL, 3X) and brine (10 mL, 2X). Organic layer was then dried over Na$_2$SO$_4$. Solvent was evaporated by rotary evaporator and dried under high vacuum. SiO$_2$ gel chromatography (CH$_2$Cl$_2$:methanol, 9.3:0.7) was performed to afford 2 (5 mg, 72%). HRMS: calc. for [C$_{30}$H$_{37}$NO$_4$Na+Na]$^+$ 498.2619, found 498.2605.

**Compound E1.2:** 1 mL of dry CH$_2$Cl$_2$ was added to a mixture of DMP (3.7 mg, 0.009 mM) and 2 (3.7 mg, 0.008 mM). The reaction mixture was stirred at room temperature for 3 h under nitrogen and 5 mL Na$_2$S$_2$O$_3$ (10%) was poured to it to stop the reaction. Organic layer was washed successively with saturated NaHCO$_3$ (5 mL, 2X), brine (5 mL, 2X) and dried over Na$_2$SO$_4$. Solvent was evaporated by rotary evaporator and dried under high vacuum. Crude mixture was used in the next step as it is. HRMS: calc. for [C$_{30}$H$_{35}$NO$_4$+H]$^+$ 474.2644, found 474.2650.

**Compound E1.3:** 3.5 mg of crude from step B was heated in sealed tube with THF:1M aqueous HCl (2:1, 2 mL) at 75 °C for 18 h. The reaction mixture was poured into 5 mL saturated NaHCO$_3$ after it was cooled down to room temperature. The aqueous layer was extracted with ether (10 mL, 2X) and successively washed with water (5 mL, 2X) and brine (5 mL, 2X). Organic layer was then evaporated by rotary evaporation and purified by SiO$_2$ gel chromatography using a glass pipette (CH$_2$Cl$_2$:methanol, 9.1:0.9) to afford 4 (3 mg, 95% 3 mg given to Dr. Kang). $^1$H NMR (CDCl$_3$, 500 MHz) δ 9.62 (t, J = 1.8 Hz, 1H), 7.19-7.05 (m, 12H), 7.02 (m, 2H), 6.77 (m, 2H), 6.66 (m, 4H), 6.60 (m, 2H), 6.44 (m, 2H), 6.21 (m, 2H), 4.07 (t, J = 5.6 Hz, 2H), 3.90 (t, J = 5.6 Hz, 2H), 2.83 (t, J = 5.6 Hz, 2H), 2.53-2.45 (m, 4H), 2.42 (s, 6H), 2.36 (s, 6H), 2.33-2.28 (m, 4H), 1.69-1.60 (m, 4H). HRMS: calc. for [C$_{28}$H$_{31}$NO$_3$+H]$^+$ 430.2382, found 430.2392 and [C$_{28}$H$_{31}$NO$_3$+2H]$^+$ 431.2460, found 431.2430.
B. **HRMS (FTCIR) Analyses — Spectra of Key Compounds**

1. **Cysteamine hexapeptide conjugate:** \( \text{HSCH}_2\text{CH}_2\text{NH–G–G–R–G–G–NHmoc} \)
   \( \text{C}_{33}\text{H}_{45}\text{N}_{10}\text{O}_8\text{S} \ [\text{M+H}^+] , \text{calcd} \ 741.3137, \text{found} \ 741.3133 \)
   
   *top = found, bottom = theoretical*

   ![Spectrum of Cysteamine Hexapeptide Conjugate](image)

2. **Hexapeptide-cypate conjugate:** \( \text{HSCH}_2\text{CH}_2\text{NH–G–G–R–G–G–NH-cypate-CO}_2\text{H} \)
   \( \text{C}_{56}\text{H}_{89}\text{N}_{12}\text{O}_7\text{S} \ [\text{M–HO}_2\text{CCH=CH}_2] , \text{calcd} \ 1053.5127, \text{found} \ 1053.5114 \)
   
   *top = found (fragment on loss of acrylic acid), bottom = theoretical (parent)*

   ![Spectrum of Hexapeptide-Cypate Conjugate](image)
3. **Trityl-cysteamine–PEG**$_{11}$–**cypate-CO$_2$H** (6b):

\[
\text{C}_{89}\text{H}_{113}\text{N}_4\text{NaO}_{16}\text{S} [\text{M+Na}^+] , \text{calcd } 1548.7770, \text{ found } 1543.8318 \\
\text{C}_{86}\text{H}_{109}\text{N}_4\text{O}_{14}\text{S} [\text{M+H–H}_2\text{C=CHCO}_2\text{H}], \text{ calcd } 1453.7661, \text{ found } 1453.7717
\]

4. **Trityl-cysteamine–PEG**$_{27}$–**cypate-CO$_2$H**:

\[
\text{C}_{121}\text{H}_{178}\text{N}_4\text{O}_{32}\text{S} [\text{M+H}^+] , \text{ calcd } 2231.2145, \text{ found } 2231.3623
\]
5. Cypate Bis-Acetal D1.1: \((\text{EtO})_2\text{HC-(CH}_2\text{)}_3\text{NH(O)C-cypate-C(O)NH-(CH}_2\text{)}_3\text{-CH(OEt)}_2\)
\(\text{C}_{57}\text{H}_{75}\text{N}_4\text{O}_6\) \([\text{M + H}^+]\), calcd 911.5681, found 911.5665

top = found; bottom = theoretical

Cypate-bis(acetal), prepared using base-free (no DIPEA) coupling conditions:
Cypate-bis(acetal) **D1.1** $^1$H NMR (CDCl$_3$, 400MHz)

Cypate-bis(acetal) **D1.1** $^{13}$C NMR (CDCl$_3$)
6. **Cypate Bis-Aldehyde D1.2**: \( \text{OHC-(CH}_2\text{)}_3\text{-NH(O)C-cypate-C(O)NH-(CH}_2\text{)}_3\text{-CHO} \)
\[ C_{49}H_{55}N_4O_4^+ [M + H]^+ , \text{calcd 763.4218, found 763.4227} \]

top = found; bottom = theoretical

HRMS of **D1.2**, taken as a MeOH solution, shows evidence of methanol hemiacetal formation (signal at 795.4487):
7. Tamoxifen-aldehyde E1.3

\[ \text{[C}_{28}\text{H}_{31}\text{NO}_3\text{+H]}^+ \text{ calcd 430.2382, found 430.2392} \]

\[^1\text{H NMR spectrum (400 MHz, CDCl}_3\text{) of tamoxifen analog E1.1}\]
8. Oxime ether conjugate D2.1; HS-C$_{12}$/PEG$_8$-ON=C-Cypate-CHO
C$_{77}$H$_{112}$N$_6$O$_{12}$S$^+$, calcd 1330.8022, found 1330.8015

![Diagram of oxime ether conjugate D2.1]
9. **Peptide aminooxy compound 2.2** (Scheme A2); \( \text{HSCH}_2\text{CH}_2\text{NHC(O)-GGRGGGONH}_2 \) 
\( \text{C}_{18}\text{H}_{35}\text{N}_{10}\text{O}_{5}\text{S}, [\text{M + H}^+]] \) calcd 535.2411, found 535.2411
10. Spectral data for intermediates in the synthesis of diblock TrS-\textbf{C}_{16}\text{PEG}_{8}\text{-NH}_2 (Scheme C2)

\textbf{a. $^1$H NMR spectrum (CDCl$_3$) of TrS-\textbf{C}_{16}\text{PEG}_{8}\text{OH}}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{hnmrspectrum.png}
\caption{$^1$H NMR spectrum (CDCl$_3$) of TrS-\textbf{C}_{16}\text{PEG}_{8}\text{OH}}
\end{figure}

\textbf{b. $^{13}$C NMR spectrum (CDCl$_3$) of TrS-\textbf{C}_{16}\text{PEG}_{8}\text{OH}}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{cnmrspectrum.png}
\caption{$^{13}$C NMR spectrum (CDCl$_3$) of TrS-\textbf{C}_{16}\text{PEG}_{8}\text{OH}}
\end{figure}
c. HRMS of TrS-C$_{16}$PEG$_8$OH; C$_{51}$H$_{80}$O$_9$S (M+Na), calcd 891.5447, found 891.5420

d. HRMS of TrS-C$_{16}$PEG$_8$NPht; C$_{59}$H$_{83}$NO$_{10}$S (M+Na), calcd 1020.5635, found 1020.5651
e. $^1$H NMR spectrum (CDCl$_3$) of TrS-C$_{16}$PEG$_8$NPhth

f. $^{13}$C NMR spectrum (CDCl$_3$) of TrS-C$_{16}$PEG$_8$NPhth
g. HRMS of TrS-C$_{16}$PEG$_{8}$NH$_2$; C$_{51}$H$_{81}$NO$_8$S, calcd 867.5682, found 868.5773 (M+H)$^+$

![HRMS spectrum](image)

h. $^1$H NMR spectrum (CDCl$_3$) of TrS-C$_{16}$PEG$_{8}$NH$_2$

![NMR spectrum](image)
11. **Target Conjugate** \(\text{HS-C}_{12}\text{PEG}_8\text{-ON=C-Cypane-C=NO-GGRRG}}\text{-C(O)NHCH}_2\text{CH}_2\text{SH} \)

\[\text{C}_{98}\text{H}_{146}\text{N}_{15}\text{O}_{19}\text{S}_{2}^{+} \left(\text{M}^+ + \text{H}^+\right)/2 \text{ calcld} 924.0164, \text{ found} 924.0161 \] (m/z where z = 2)

Sample prepared by SL (notebook ref: SL-III-5)